STAPHYLOCOCCAL ENTEROTOXIN SUPERANTIGENS: STRUCTURAL STUDIES AND ROLE IN AUTOIMMUNE DISEASE

By

JEANNE MARGARET SOOS

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I would like to dedicate this work to my parents, Steven and Elizabeth Soos. My father has believed in me and helped me to believe in myself. My mother's caring, intelligence and strength and the aspirations she had for her daughter will always be an inspiration to me.

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STAPHYLOCOCCAL ENTEROTOXIN SUPERANTIGENS: STRUCTURAL STUDIES AND ROLE IN AUTOIMMUNE DISEASE

By

Jeanne Margaret Soos

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The staphylococcal enterotoxins have profound effects on the immune system, causing massive T cell stimulation and cytokine release. We have studied the interaction of toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin B (SEB) with the major histocompatibility complex (MHC) class II molecule, the role of superantigens in experimental allergic encephalomyelitis (EAE) and the effect of type I interferon (IFN) on superantigen-induced stimulation. In the structural studies, overlapping peptides of TSST-1 were synthesized and peptides (39-68) and (155-194) were found to compete with TSST-1 for binding to Raji and A20 cells. SEB peptides were also synthesized and competition with SEB

was examined on Raji and DR1 transfected L cells. Peptides (1-33), (31-64) and (179-212) were effective competitors on DR1 transfected L cells while peptides (1-33), (124-154), (150-183) and (179-212) were effective competitors on Raji cells. These findings provide insight into superantigen interaction with MHC class II molecules.

The observation that superantigens stimulate T cells and in some cases anergize and delete Vß specific T cell subsets has raised speculation that superantigens may play a role in autoimmune disease. We first examined the ability of SEB to prevent EAE, a murine autoimmune model for multiple sclerosis (MS). PL/J mice treated with SEB prior to immunization with myelin basic protein (MBP), the autoantigen involved in EAE and MS, were protected against development of EAE. Study of the protected mice revealed that the T cell subset normally responsible for disease had been depleted and anergized. In contrast, we examined the effect of superantigen after immunization with MBP. We found that both SEB and SEA were able to reactivate EAE in resolved and asymptomatic mice. Thus, superantigen is able to both prevent and reactivate EAE, depending on the time of superantigen treatment relative to immunization with MBP. In an effort to identify potential therapeutics for superantigen associated disease, the type I IFNs, α, ß, and τ, were shown to inhibit superantigen-induced T cell stimulation. In all, superantigens and their derivatives may offer a mechanism by which to modulate autoimmune responses. The effects of the type I interferons on superantigens may provide yet another level of control over such diseases.

INTRODUCTION

Overview

Superantigens are among the most potent T cell activators known (Langford et al., 1978). They stimulate as many as 1 in 5 T cells as compared to classical peptide antigens which stimulate as few as 1 in 10,000 T cells. These unique molecules are produced by both bacteria and viruses and are presented in Table I. The prototype for the bacterial superantigens is the family of staphylococcal enterotoxins. Original studies of these superantigens have been the basis for the characterization of more recently described superantigens. In addition to the staphylococci, other organisms that have been shown to produce superantigens are the group A streptococci, *Mycoplasma arthritidis, Mycobacterium tuberculosis, Yersinia pestis,* and *Clostridium perfringens.* Like some bacteria, certain viruses are also able to produce superantigens. The first identified virus was the mouse mammary tumor virus (MMTV), which has served as the prototype for the viral superantigens. Before its description as a viral product, these superantigens were thought to be involved as self-superantigens involved in thymic education or as coligands for higher affinity interaction of certain immune cells (Pullen et al., 1988; Janeway et al., 1989). Other viruses that have been shown to possess superantigenic activity are mouse leukemia virus, human spumaretrovirus, rabies, and Epstein-Barr virus. Many of the bacterial superantigens have been shown to

Table I. The superantigens.

Bacterial

Prototype: Staphylococcal enterotoxins

Organism	Protein
Staphylococcus aureus	Enterotoxins
Group A streptococci	Pyrogenic exotoxins
Mycoplasma arthriditis	T cell mitogen
Mycobacterium tuberculosis	Not identified
Yersinia pestis	Not identified
Clostridium perfringens	Exotoxin

Viral

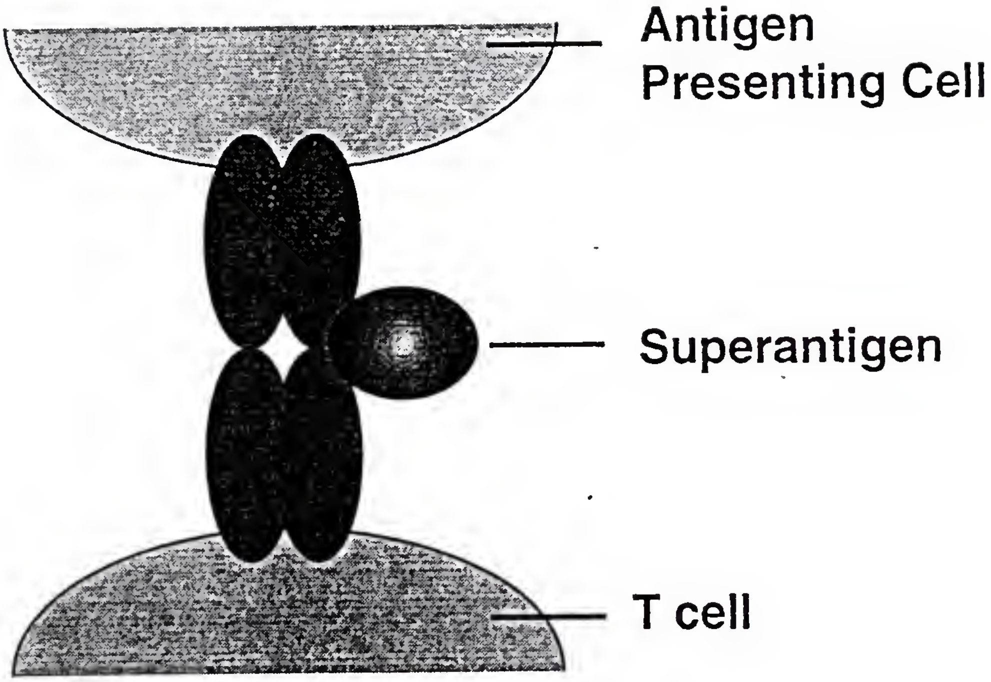
Prototype: Superantigen from Mouse Mammary Tumor Virus (MMTV)

Virus	Protein
Mouse mammary tumor virus - type B retrovirus	ORF product
Mouse leukemia virus - type C retrovirus	Gag protein
Human spumaretrovirus - foamy virus	bel 3 gene product
Rabies	nucleocapsid protein
Epstein-Barr virus	Not identified

play a role in disease caused by their organisms of origin. These maladies include food poisoning, toxic shock syndrome, and Kawasaki syndrome. They may also play a role as environmental factors in the relapsing-remitting nature of certain autoimmune diseases. In the case of the viral superantigens, only MMTV's role in disease is clear, as it has been shown to be important in virus life cycle. There has been much speculation concerning the purpose of the other viral superantigens; however, direct evidence is as yet unavailable.

Recently, the mechanisms by which the bacterial superantigens exert their effects have been greatly clarified. It has been determined that these molecules are presented by MHC class II antigens to T cells. However, their mode of presentation is quite different from that of classical peptide antigen in that the superantigens bind to the outside of the antigen binding groove, do not require processing prior to presentation and exhibit very little MHC haplotype restriction (Carlsson et al., 1988; Fleischer and Schrezenmeier, 1988; Mollick et al., 1989; Dellabonna et al., 1990). The recognition of superantigen by the TCR is also unusual. Superantigens bind to the variable region of the ß chain of the TCR and thus stimulate T cells in a Vß specific manner (White et al., 1989; Gascoigne and Ames, 1991). The MHC/superantigen/TCR trimolecular complex is depicted in Figure 1. Vß specific stimulation explains why the superantigens are able to activate as much as 20 % of a T cell repertoire. It has been shown that the different superantigens exhibit varying Vß specificities. T cell stimulation in this manner results in massive cytokine release, including interleukin-2 (IL-2),

Figure 1. The MHC/superantigen/TCR trimolecular complex.


Antigen
Presenting Cell
Superantigen
T cell

interferonγ (IFNγ), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1). Under certain circumstances, superantigens are also able to anergize and/or delete specific Vß T cell subsets subsequent to initial T cell activation in naive animals (Kawabe and Ochi, 1990; Rellahan et al., 1990; Kawabe and Ochi, 1991).

The viral superantigens are also capable of the functions described above for the bacterial superantigens. A stumbling block in the study of the viral superantigens has been the difficulty of their isolation. Thus, protein characterization, in terms of direct binding studies, for example, has remained elusive. Clearly the superantigens, both bacterial and viral, have profound effects on the immune systems of their victims, and the elucidation of their mechanisms of action will enable the development of therapeutics for many of the syndromes for which they are responsible.

Staphylococcal Enterotoxin Superantigens

Structure

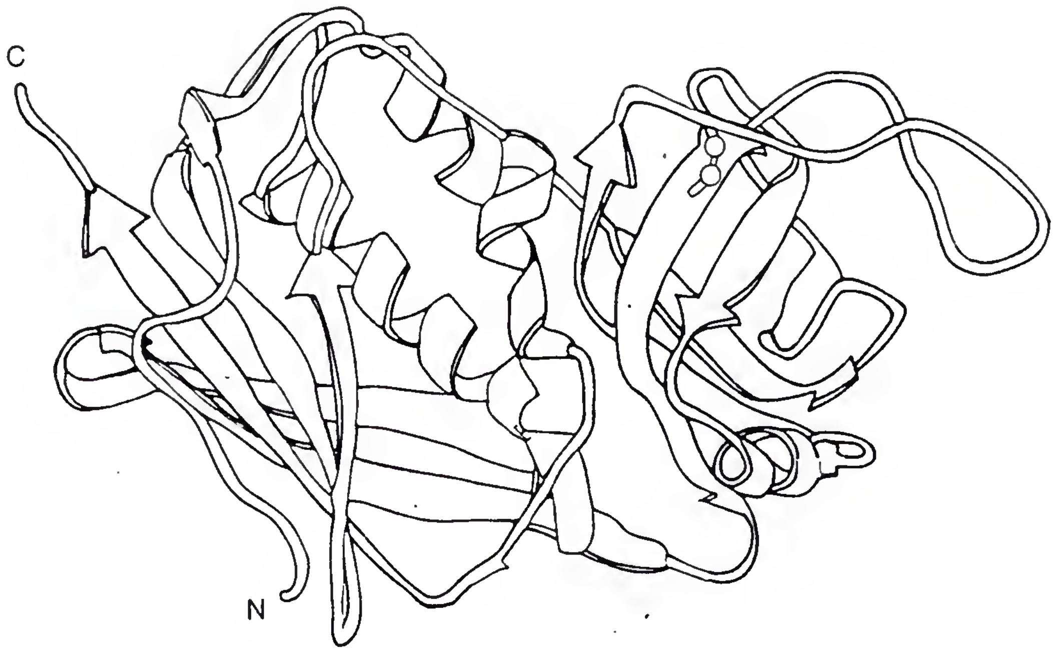
The staphylococcal enterotoxins are a family of structurally related proteins that are single chain molecules with molecular weights of approximately 24 to 30 kDa. They are acid and heat stable and rich in threonine, serine and aspartic acid residues. Thet are also charged with pIs ranging from 7.0 to 8.6. All of the members of the staphylococcal enterotoxin family, with the exception of TSST-1, contain a centrally located disulfide loop, the function of which remains in question. In the cases of SEA, SEB and SEC, enzymatic cleavage in the region of the disulfide bridge had no effect on biological activity (Spero et al., 1973; Noskova et al., 1984). However, the stimulatory activities of these toxins based on the disulfide bridge have been dissociated for T cells and monocytes (Grossman et al., 1990). The reduction and alkylation of the staphylococcal enterotoxins affected T cell activation but had no effect on monocyte production of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). The staphylococcal enterotoxins have been serologically classified into five groups A through E, with SEC divided further into three subtypes on the basis of minor epitopes (Bergdoll, 1985). Another member of this toxin family was originally designated SEF but was later renamed TSST-1 (Bergdoll, 1985). The amino acid sequences of the staphylococcal enterotoxins exhibit appreciable homology (Betley and Melakanos, 1988). SEA, SEE and SED are related in amino acid sequence, while SEB and SEC share greater homology with each other. Two regions of these toxins, residues 106 to residue 119 and residue 147 to residues 163, have been identified as highly conserved (Betley and Melakanos, 1988; Iandolo, 1989).

While sequence homology of the staphylococcal enterotoxin family members range from as little as 30 % to as much as 86 %, all of the toxins regardless of homology exhibit similar biological activity (Betley and Melakanos, 1988). Such common biological function suggests that their activity is based on similar secondary and tertiary structures. Initial studies using such techniques as circular dichroism (CD) and tryptophan quenching found that the staphylococcal enterotoxins characteristically have low α helical content together with a high content of ß structure (Singh et al., 1988a). In general, the more closely related toxins exhibit greater similarity in their CD spectra. It is of interest that TSST-1, which shares only minimal sequence homology, also has the low α helix and high ß sheet content characteristic of the other staphylococcal enterotoxins (Singh et al., 1988b). This argues strongly that the similar functions of these toxins are maintained at the secondary and tertiary structural levels.

Recently, the three dimensional crystal structures of SEB and TSST-1 have been determined (Swaminathan et al., 1992; Acharya et al., 1994). In the case of SEB, the structure consists of a main chain fold containing two domains, which suggests a general motif for the other enterotoxins (Figure 2). The first domain is composed of residues 1 to residue 120 and contains two ß sheets and three α helices. Two of the ß stands form a cylindrical barrel forming a crisscross pattern. The disulfide loop is present in this domain in residues 99 to 105 extending into the solvent. The second domain is composed of residue 127 to residue 239 and contains 2 α helices and 7 ß strands. The second domain is more complicated and actually contains an infrequently observed left-handed crossover of ß strands 6 and 12.

A shallow cavity, formed by the two structural domains of SEB, is considered to be a binding site for the TCR. This is supported by a previous study that identified residues important for TCR binding (Kappler et al., 1992), and it has been shown that these residues lie along the sides of the cavity. Upon close inspection of the structure, three adjacent loops at the edge of the cavity appear to collectively form a structural unit important for TCR interaction. Of the 21

Figure 2. A ribbon model of the three dimensional crystal structure of SEB.

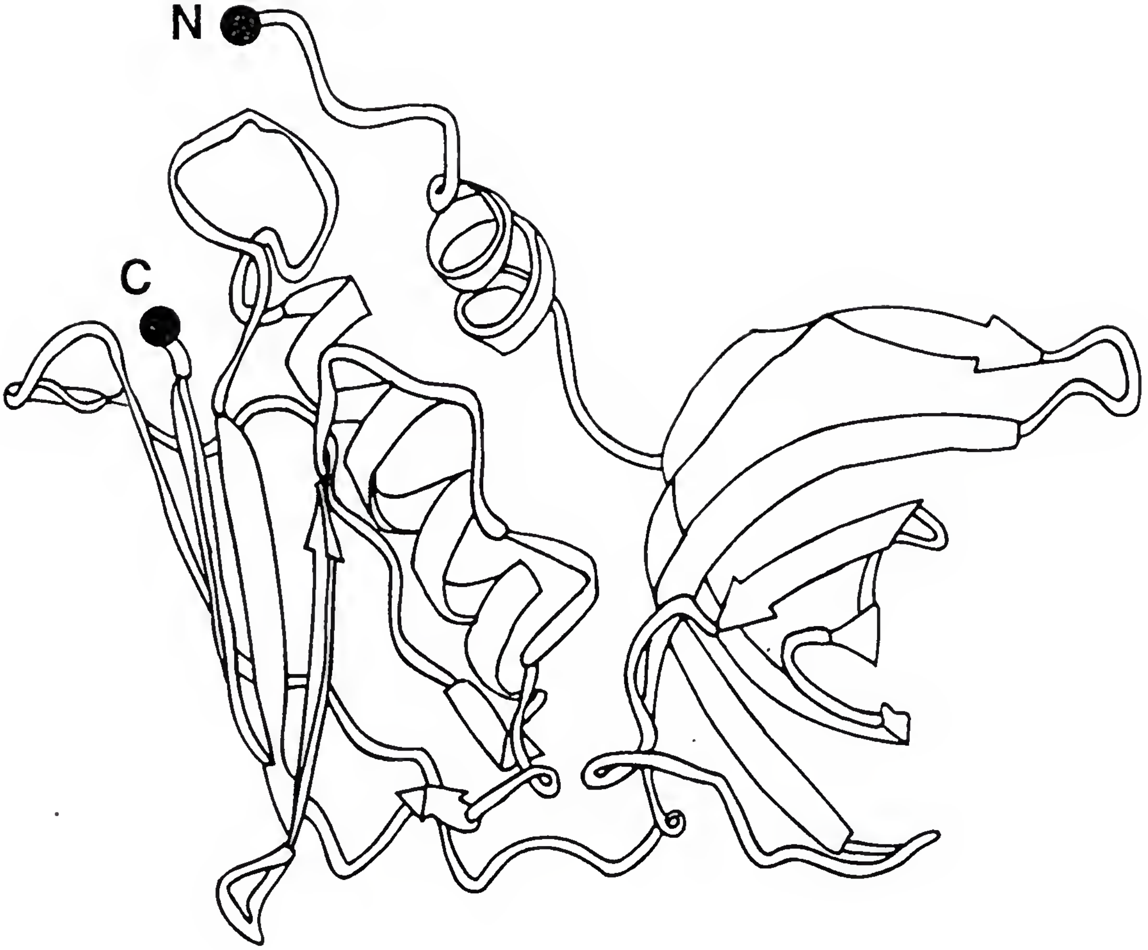

C
N

residues of SEB suggested to be important for TCR binding, 18 residues are conserved between SEB and SEC1. Thus, it makes sense that these two superantigens stimulate the same Vß specific TCRs with the exception of one.

The TSST-1 structure is a monomeric, two domain, compact molecule (Figure 3). The N-terminal domain of TSST-1 is composed of an α helix and multiple polypeptide folds of ß strands that form a continuous "roll" or what is known as a ß-barrel. An extended chain caps an end of the ß-barrel. The C-terminal domain is composed of one relatively long α-helix, which is cupped by a highly twisted ß-sheet forming an elaborate ß-grasp motif (Murzin, 1992). The α-helix present in the C-terminal domain appears to serve as the backbone of the molecule.

The topology of TSST-1, as determined by its three dimensional structure, is similar to the structure of SEB in that it is composed of two domains and exhibits a high content of ß-sheet relative to α-helix. However, a number of differences in the TSST-1 and SEB structures exist. Among these differences are 1) the topology of the TSST-1 C-terminus is unique compared to SEB as it contains a ß-grasp motif; 2) TSST-1 is extensively truncated in a number of loop regions of SEB, including the cysteine loop, which is consistent with the fact that TSST-1 does not contain a disulfide bridge like the other staphylococcal enterotoxins; 3) there is no structural equivalent of the N-terminus of SEB in the TSST-1 structure; and 4) the TSST-1 structure contains even fewer α-helices than the SEB structure. Thus, a general motif is apparent from the SEB and TSST-1 3-dimensional structures, but

Figure 3. A ribbon model of the three dimensional crystal structure of TSST-1. The blue and red balls represent N and C termini, respectively.


N
C

certain structural features that appear important for SEB are not present in TSST-1 probably due to the lack of TSST-1 sequence identity with the other staphylococcal enterotoxins.

Studies are presently underway for the high-resolution determination of the MHC/superantigen/TCR trimolecular complex (Brown et al.,1993). It has been suggested that the MHC and superantigen molecules involved in this study are HLA-DR1 and SEB. If such information is obtained, it will prove extremely useful in understanding the superantigen mechanism of pathogenicity and provide a strong basis for the development of engineered therapeutics that can target specific epitopes identified from the sites of interaction within the trimolecular complex.

Function

The first observation that a staphylococcal enterotoxin, namely SEB, was able to induce mitogenesis of human peripheral lymphocytes was made in 1970 (Peavy et al., 1970). The staphylococcal enterotoxins were subsequently shown to be specific for the activation of T cells (Johnson and Bukovic, 1975). In addition to their ability to induce mitogenesis in T cells, they are potent inducers of a number of cytokines, including IFNγ, IL-2 and TNF (Langford et al., 1978; Carlsson and Sjogren, 1985) and have been implicated in the suppression of antibody responses and certain T cell responses (Torres et al., 1982; Papermaster et al., 1983). In order to induce activation, the staphylococcal enterotoxins are required in amazingly low concentrations (Langford et al., 1978). For example, SEA, the most potent of the staphylococcal enterotoxins, is able to stimulate maximal DNA synthesis and production of IFNγ at concentrations of 3.5×10^{-13} and 3.5×10^{-10} M respectively (Langford et al., 1978), making the staphylococcal enterotoxins among the most powerful T cell activators known.

During the 1980s, further characterization of superantigen-induced T cell activation continued. The first clue that the microbial superantigens were actually a type of antigen came when it was discovered that antigen presenting cells and more specifically, the MHC class II molecules on their surface were required for presentation of these unique antigens to T cells (Carlsson et al., 1988; Fleischer and Schrezenmeier, 1988; Mollick et al., 1989). (The term superantigen was later given to these enterotoxins.) However, the manner in which the MHC class II molecule presents superantigen is quite different from that of a classical peptide antigen. First, presentation of microbial superantigens exhibits very little MHC haplotype restriction. Second, the microbial superantigens do not require processing prior to presentation by MHC class II molecules as evidenced by the fact that paraformaldehyde fixed APCs are as proficient in presenting superantigen as their untreated counterparts. Third, superantigens have been shown to bind to the outside of the antigen binding groove of MHC class II molecules, in contrast to nominal peptide antigens which are known to bind inside the groove (Dellabonna et al., 1990; Russell et al., 1990). Thus, the manner in which superantigens are presented by the MHC class II molecule proved to be quite unique and provided insight into how they are recognized by T cells.

If the binding of superantigen to MHC class II molecules is so unusual, then how are the superantigens recognized by the TCR? With the advent of monoclonal antibodies specific for the Vß elements of the TCR, much insight was gained about how the microbial superantigens stimulate T cells. It was shown in vitro that SEB stimulated specific T cell subsets based upon the Vß region of the TCR (White et al., 1989). For example, SEB was shown to stimulate murine T cells bearing Vßs 3, 7, 8, 11, and 17 (Callahan et al., 1989). This Vß specific T cell stimulation was also shown to hold true in the human system (Kappler et al.,1989). In addition to in vitro experiments, one of the most interesting pieces of evidence in the stimulation of human Vßs came from studies of patients suffering from toxic shock syndrome. A number of years earlier, it had been shown that the pathogen responsible for this sometimes fatal disease was a newly isolated strain of *Staphylococcus aureus* that produced a toxin aptly titled toxic shock syndrome toxin-1 (TSST-1) (Bergdoll et al., 1981). Patients infected with TSST-1 producing *S. aureus,* often via tampon use, exhibited as great as a 60 % expansion of their Vß 2 bearing T cells in both the CD4 and CD8 subsets during the acute phase of infection (Abe et al., 1992). The levels of Vß 2 bearing T cells remained elevated until resolution of disease symptoms when the expanded subset exhibited a gradual return toward normal levels. Subsequent studies have determined the Vß specificities of the microbial superantigens in both the murine and human systems (Table II).

Table II. Vß specificities of some microbial superantigens.

	Vß specificity	
Toxin	Mouse	Human
SEA	1, 3, 10, 11, 17	1, 5, 6's, 7.3-7.4, 9.1
SEB	7, 8.1-8.3, 11, 17	3, 12, 14, 15, 17, 20
SEC1	3, 8.2, 8.3, 11, 17	3, 6.4, 6.9, 12, 15
SEC2	3, 8.2, 10, 17	12, 13.2, 14, 15, 17, 20
SEC3	7, 8.2	3, 5, 12, 13.2
SED	3, 11, 17	5, 12
SEE	11, 15, 17	5.1, 6's, 8, 18
TSST-1	15, 16	2
ExFT	10, 11, 15	2
SPE-A	ND	8, 12, 14, 15
SPE-B	ND	2, 8
SPE-C	ND	1, 2, 5.1, 10
MAM	5.1, 6, 8.1-8.3	3, 17

ND-not determined or undefined.

For some time the only immunomodulatory activities of the microbial superantigens known were their ability to stimulate T cells and their involvement in certain immune suppression responses (Papermaster et al. 1983). In addition to these, they are also capable of inducing anergy (rendering T cells nonfunctional) and depletion (reduction in the number of T cells) of Vß specific T cell subsets. Original studies of induction of specific clonal anergy by SEB were shown in vitro and in vivo and were the first examples of peripheral T cell tolerance (Kawabe and Ochi, 1990; Rellahan et al., 1990). Studies in vitro showed that a number of staphylococcal enterotoxins including SEA, SEB, SEC1-3, and SED were able to induce specific clonal anergy (O'Hehir and Lamb, 1990). Cloned human T cells specific for hemagglutinin (307-319) were exposed to the various staphylococcal enterotoxins for a 16 hour period and then challenged with peptide antigen. The T cells failed to respond to their natural ligand when pretreated with superantigen even in the presence of exogenous IL-2.

It was shown in vivo that adult mice administered SEB exhibited a profound state of anergy in their Vß8 T cell subset. Such anergy was evidenced by their inability to proliferate to subsequent in vitro stimulation with either SEB or anti-Vß8 antibodies. Vß8 T cells from SEB primed mice also failed to proliferate in response to exogenous IL-2 indicating a defect in their IL-2 responsiveness. This same T cell subset also exhibited reduced activity in primary cytotoxicity assays. Induction of anergy by SEB was shown to occur primarily in the Vß8 CD4 T cell subset but not in the CD8 subset. In these studies, anergy induced by SEB was shown to last at least two weeks while data presented in this dissertation suggest that abrogation of response to SEB may last beyond 40 days after initial exposure to superantigen. It is important to point out that superantigen-induced anergy occurs in adult naive mice. However, mice that have been primed with another antigen prior to exposure to SEB may not be so readily anergized. Data presented in this dissertation show that mice immunized with another antigen, in this case myelin basic protein (MBP) in complete Freunds adjuvant (CFA) or CFA alone, are not susceptible to the induction of anergy in their Vß8 T cell subset. Thus, it appears that the circumstances and conditions of the subject can influence the induction of anergy by superantigen.

Deletion of Vß8 CD4 T cells in adult naive mice primed with SEB has also been observed (Kawabe and Ochi, 1991). Mice exposed to SEB first exhibit an increase in both the Vß8 CD4 and CD8 subsets on days one through four after exposure. By day seven the Vß8 CD8 subset returned to near normal levels. In contrast, the levels of Vß8 CD4 T cells were reduced by half of normal prior to exposure to SEB. Deletion by superantigen was determined to occur in the periphery as a reduction in the Vß8 CD4 subsets occurred in both normal and thymectomized mice. The mechanism of deletion was determined to occur via programmed cell death or apoptosis. By day four after exposure to SEB, the Vß8 CD4 subset, while still detectable by flow cytometry, exhibited genomic DNA fragmentation associated with programmed death. Extremely low concentrations of superantigens are also able to delete Vß specific T cells although the events prior to deletion are different compared to deletion induced by higher doses of superantigen. When mice are chronically exposed to very low doses of superantigen, i.e., multiple injections within a defined time period, the initial T cell stimulation observed in previous deletion studies was not seen. Instead dramatic deletion of the target subsets was observed (McCormack et al., 1993). Thus dosage of superantigen may also greatly influence the course of deletion of specific Vß T cells.

The critical determinant leading to clonal deletion has been shown to be one of the cytokines strongly induced by superantigen, namely IL-2 (Lenardo, 1991). Experiments with the antigen specific cell line, A.E7, showed that exposure to IL-2 prior to antigen specific stimulation results in programmed cell death, while exposure to IL-2 after antigen specific stimulation results in proliferation. In mice injected with SEB, an anti-IL-2 antibody that blocks binding to the IL-2 receptor α chain inhibited the marked reduction of Vß8 T cells normally observed in previous deletion studies. Therefore, superantigen-induced IL-2 is integral to Vß specific T cell deletion. It would appear that anergy and deletion of Vß specific T cells by superantigen occurs concomitantly with approximately 50 % depletion of a Vß specific subset with the remainder of the subset undergoing anergy. Whether the entire remainder of the subset is anergized or if certain cells are able to escape anergy remains to be determined.

Only limited study of the anergy and deletion properties of the microbial superantigens in humans has been conducted. To date, no studies of patients suffering from food poisoning have been undertaken. In the case of toxic shock syndrome, after expansion of the Vß2 subset occurs, levels of Vß2 bearing T cells return to normal. No depletion of Vß2 bearing T cells was observed at any time point after disease resolution. The ability of superantigen to induce anergy and/or deletion in the human system remains to be studied in depth and much may depend on the timing of exposure and dosage of superantigen.

Superantigen Interaction with MHC Class II Molecules

Although the staphylococcal enterotoxins had been determined to be responsible for a large percentage of food poisoning cases, their mode of action has remained in question for some time. This was partly resolved by studies showing that SEA bound to murine lymphocytes (Buxser et al., 1981). SEA was shown to bind to a single class of receptor, and via inhibition studies it was also determined that SEA and SEE bound to the same receptor. Subsequent to this study it was shown that paraformaldehyde fixed antigen presenting cells were as efficient in presenting the bacterial toxins as were untreated cells suggesting that the enterotoxins do not require processing prior to presentation (Carlsson et al., 1988). The specific receptors for the staphylococcal enterotoxins on antigen presenting cells were shown to be the MHC class II molecule by a number of studies including 1) use of anti-MHC antibodies for inhibition of binding (Fleischer and Schrezenmeier 1988), 2) direct binding to purified MHC class II molecules (Lee and Watts, 1990), 3) genetic analysis illustrating the necessity of I-E expression (Cole et al., 1981), and 4) use of MHC class II negative cells transfected with MHC class II genes (White et al., 1989). Quantitative binding studies with a number of the staphylococcal enterotoxins have determined their affinities for HLA and MHC class II molecules (Buxser et al., 1981; Fraser, 1989; Mourad et al., 1989). In general, the staphylococcal enterotoxins have a higher affinity for the HLA molecules than for the MHC class II molecules. Of all the staphylococcal enterotoxins, SEA exhibits the highest affinity for the class II molecules in both the HLA and MHC systems, which may assist in explaining why SEA is the most potent of all of the staphylococcal enterotoxins. Further characterization of superantigen MHC interaction showed that this binding is quite unusual compared to classical peptide antigen. In addition to the lack of processing, superantigens bind to the outside of the antigen binding groove (Dellabona et al., 1990). The toxins also exhibit a lack of MHC restriction. However, it has been shown that alleles of HLA-DR differ in their ability to present the staphylococcal enterotoxins to T cells (Herman et al., 1990). Thus, while there is lack of MHC restriction, the enterotoxins can exhibit haplotype preference.

A number of questions have arisen regarding the requirement of MHC binding by superantigen and the ultimate stimulation of Vß specific T cell subsets. Do MHC and TCR interactions influence superantigenic stimulation or does binding to MHC class II induce a conformational change in the superantigen that allows for binding to the TCR? In the cases of two superantigens, SEA and SEE, it has been shown that zinc is required for the stabilization of the binding domain of MHC class II molecules but is not directly involved in the interaction with the MHC class II molecule (Fraser et al., 1992). The experiments involving zinc as a regulatory factor provide insight into the mode of action of these two superantigens. Binding of SEA and SEE, but not SEB or TSST-1, was abolished by EDTA and reconstituted in the presence of Zn^{2+}. Zinc binding appears to be an essential first step in the formation of the MHC class II binding domain of SEA and SEE. That zinc is able to produce an active site on SEA for MHC class II binding suggests that SEA is not a wholly inflexible molecule as previously thought due to its total resistance to protease and heat. Unlike SEA, TSST-1 did not require zinc for MHC class II binding, which may be directly related to the more severe consequences of toxic shock syndrome compared to food poisoning. The involvement of zinc and the requirement of MHC class II for binding of superantigen to the Vß region of the TCR suggests two possible explanations. The first of these is that binding of superantigen to MHC class II induced some type of structural modification that reveals a cryptic site for interaction with the TCR. The second is that superantigen binding induces a modification of the MHC class II molecule required for high affinity interaction with the TCR Vß region.

Binding regions of the MHC class II molecule involved interaction with the staphylococcal enterotoxins have been the subject of intense study. Competitive binding studies have determined that TSST and SEB bind to two different sites on HLA-DR. Both of these sites are also bound by SEA, SED, and SEE (Scholl et al., 1989; Scholl et al., 1990; Chintagumpala et al., 1991; Pontzer et al., 1991b). It is of interest that SEB and TSST-1 are not closely related by amino acid sequence while SEA, SED and SEE, all of which bind to the same sites, share more that 70 % amino acid sequence homology. Several studies have suggested that there are approximately three distinct, although possibly overlapping, binding sites on HLA-DR for these bacterial toxins. Further examination of superantigen/MHC class II interaction has revealed the specific binding sites for a number of the staphylococcal enterotoxins. SEA, via direct involvement of its N-terminus, was shown to bind to region 65 through 85 of the ß chain of I-A^b (Russell et al., 1990). Sites in this region of the ß-chain, specifically amino acid residues 72, 80 and 81, were shown to be important for SEA binding by the synthetic peptide approach and site directed mutagenesis of DR transfected L929 cells (Russell et al., 1991; Herman et al., 1991). The α chain of the I-A^b molecule was also shown to be involved in SEA binding (Russell et al., 1991). Studies of SEE binding also point to the importance of histidine 81 of the ß chain of HLA-DR (Karp and Long, 1992). In the case of TSST-1, both the α and ß chains of both HLA and MHC class II molecules have been shown to contribute to binding of this toxin (Braunstein et al., 1992). Interpretation of these studies suggests that residues of the two helices together define the binding site for TSST-1 and that only one binding site for TSST-1 is present on a single class II molecule. This is supported by the crystal structure of TSST-1 (Acharya et al., 1994). Thus, multiple superantigen binding regions are present on the MHC class II molecules.

Superantigen Interaction with TCR and Accessory Molecules

The manner in which superantigens stimulate T cells is unique in that they cause the activation of many or all T cells bearing specific TCR ß chain variable region elements (White et al., 1989). Superantigen interaction with the TCR has been of interest, however, such studies have proved more difficult due to the requirement of superantigen binding to MHC class II prior to recognition by the TCR. Initial studies involved the production of a chimeric TCR in which products of human Vß genes were introduced into a murine TCR expressed on the surface of a mouse T cell hybridoma (Choi et al., 1990a). Residues 67 through 77 of human Vß 13.2 were determined to be important for the function of SEC2. Direct binding of superantigen to the TCR was demonstrated by the use of a secreted form of the TCR ß chain of Vß 3 specificity (Gascoigne and Ames, 1991). SEA, in the presence of Raji cells, was shown to bind directly to the secreted ß chain and was dependent on the presence of the MHC class II molecule as SEA did not bind the ß chain in the absence of Raji cells. This was the first report of direct TCR binding with a natural ligand and provided clear evidence that presentation of superantigen by MHC is a requirement for interaction with the Vß region of the TCR. Studies to define the region of the variable portion of the ß chain have identified amino acid residues 57 through 77 of Vß3 of the TCR as being responsible for SEA/TCR interaction (Pontzer et al., 1992). These residues lie in the CDR4 region of the TCR. The CDR4 region forms part of the side of the ß chain, exposed to the aqueous phase and well away from the classical peptide antigen/MHC class II binding site or any site of interaction between the Vα and Vß chains. Thus, the trimolecular complex appears to involve MHC and TCR interaction with the superantigen on the external sides of the MHC ß chain α helix with amino acids between residues 57 and 77 of the TCR.

Interestingly, the observation has been made that there is only a limited requirement for accessory molecules on T cells for staphylococcal enterotoxin stimulation. Clearly, CD4 and CD8 molecules do not play a role, as T cell clones and hybridomas lacking these two accessory molecules usually respond to the bacterial superantigens (Fleischer and Schrezenmeier, 1988; Fleischer and Schrezenmeier, 1989). The one set of accessory molecules that has been shown to be directly involved in superantigen stimulation is ICAM-1/LFA-1 (van Seventer et al., 1991). This was evidenced by the use of a solid matrix assay in which soluble MHC class II, SEA and ICAM-1 were present and T cell stimulation was measured. In the absence of soluble ICAM-1, SEA-induced stimulation did not occur. When soluble ICAM-1 was added to the solid matrix assay, SEA-induced stimulation of T cells was close to that of previously observed levels under normal conditions. The role of another set of accessory molecules, B7/CD28, remains uncertain as a number of conflicting reports have been made concerning their effect on superantigen-induced activation (Fischer et al., 1992; Green et al., 1992; Damle et al., 1993).

Mls and the Viral Superantigens

The endogenous superantigens originally termed minor lymphocyte stimulatory (Mls) determinants were first detected based on their ability to stimulate a strong, primary mixed lymphocyte reaction (MLR) between cells from mice bearing the same MHC haplotype (Festenstein, 1973). In the MLR, naive T cells were shown to respond to Mls determinants presented by MHC class II molecules on the surface of various cell types (Peck et al., 1977). This stimulation by Mls was shown to be greater than that induced by the products of MHC in a primary MLR (Wilson et al., 1968; Janeway et al., 1980; Lutz et al., 1981; Miller and Stutman, 1982). Original studies of Mls murine strain distribution suggested that the Mls locus expressed four alleles, a, b, c, and d, and that these alleles encoded polymorphic determinants with variable stimulatory activity (Festenstein, 1974). Other non-MHC related determinants were also designated as Mls determinants based upon their MLR stimulatory activity such as Mls^x in PL/J and Mls^e in C3H/Tif (Coutinho et al., 1977; Janeway and Katz, 1985).

With the advent of monoclonal antibodies specific for the Vß elements of the TCR, important information was gained about the mechanism by which the Mls determinants induce T cell stimulation. It was shown in vitro that naive T cells bearing particular Vß elements respond to Mls determinants and that different Mls determinants were specific for particular Vßs. In contrast, in vivo studies showed that T cells bearing specific Vßs were clonally deleted during development in the thymus of mice expressing particular Mls determinants (Blackman et al., 1990). The observation that Mls determinants could clonally delete thymic T cells led to much speculation and raised the question of Mls determinants as self superantigens. The self superantigen hypothesis suggested that mice expressing self superantigens would remove responsive T cells from the periphery; this elimination would occur via clonal deletion during thymic education resulting in self tolerance. It had been shown that EAE and induced rheumatoid arthritis were chiefly caused by T cells expressing Vß8.2 and Vß6, respectively (Acha-Orbea et al., 1988). Thus, the deletion of Vß specific T cell subsets by self superantigens could confer protection against autoimmunity as well as the ill effects of bacterial toxins. Another hypothesis on the function of Mls was that the as yet unidentified Mls protein served as a coligand between the TCR and MHC, its purpose being to potentiate T cell responses, allowing such responses to occur more rapidly (Janeway, 1990). The nagging question that remained was why had structures similar to Mls not been detected in humans. Ultimately, the answer to the true identity of the Mls determinants proved somewhat surprising.

The Mls determinants are actually a collection of products of the 3' open reading frame encoded in the long terminal repeat of the murine retrovirus, mouse mammary tumor virus (MMTV) (Dyson et al., 1991; Frankel et al., 1991; Marrack et al., 1991; Woodland et al., 1991). MMTVs are known to be type B retroviruses responsible for the induction and transmission of mammary carcinoma in mice (Heston et al., 1945). Endogenous MMTV proviruses are present in the germ line of all inbred mice. Different strains of mice harbor distinct proviruses at multiple locations in their genome. Many are defective and unable to produce infectious viruses while some can be activated in the mammary gland, resulting in the shedding of viral particles into milk (Kozak et al., 1987). Thus, the transmission of MMTV superantigen can occur by both inheritance and milk-borne infection.

The structural characterization of the MMTV superantigen, MMTV-7, suggests that it is probably a 45K type II integral membrane protein with an intracellular N terminus and an extracellular, glycosylated C terminus (Choi et al., 1992; Korman et al., 1992). Gene truncation experiments showed that the N terminus was important to the activity of MMTV-7 but its function was unclear (Choi et al., 1992). Subsequently, it was shown that the MMTV-7 superantigen may be synthesized as a precursor protein, undergo proteolytic cleavage and be expressed as an 18.5K surface protein (Winslow et al., 1992). Such proteolytic cleavage would involve the removal of the N-terminal transmembrane domain resulting in the expression of the C-terminal residues that are somehow "tethered" to the membrane. Such tethering to the cell surface may occur to MHC class II or the N terminal portion of MMTV-7 via noncovalent associations or via covalent lipid addition. However, it has been shown that intracellular transfer of MMTV-7 does take place, but how the transfer of a type II integral membrane protein or tethered protein occurs is difficult to explain. One potential explanation is that the mature, functional form of the viral superantigen protein is a soluble protein although this has yet to be proven. While MMTV-7 superantigen has been detected on the surface of B cells, the only studies that have shown direct binding of MMTV-7 have employed the synthetic peptide approach. It was shown that residues 76 through 119 of MMTV-7 are responsible for binding to MHC class II and that MMTV-7 shares an MHC class II binding site with the bacterial superantigen, SEA (Torres et al., 1993). In this study, the labelled MMTV-7 peptide

ORF(76-119) was shown to bind directly to murine B cells and an MHC class II peptide encompassing the alpha helical residues 60 through 90 of the beta chain. Using antipeptide antibodies, the immediate C-terminal residues 310 through 322 of MMTV-7 were found to be important for interaction with the TCR (Winslow et al., 1992). While recent studies have provided information regarding regions important for binding and function, direct evidence as to the mature form of the MMTV-7 protein remains elusive.

Experimental Allergic Encephalomyelitis

The animal model experimental allergic encephalomyelitis (EAE) is a prototype for antigen specific T cell mediated autoimmune disease (Gonatas and Howard, 1974; Ortiz-Ortiz and Weigle, 1976). The primary autoantigen involved in the induction of EAE is myelin basic protein (MBP). MBP is a predominant protein present in myelin in the central nervous system (CNS). The mediators of pathogenesis in EAE are MHC class II restricted, CD4 T lymphocytes that are MBP specific. EAE can be induced in a number of species, and certain forms are characterized by relapsing paralysis. Histopathology studies have demonstrated the infiltration of perivascular lymphocytes and demyelination in the CNS. The characteristics of EAE suggest it as the primary model for the human demyelinating disease, multiple sclerosis (MS) (Alvord, 1984; Raine, 1983).

The first description of EAE was made when adverse allergic responses to the original rabies vaccine developed by Louis Pasteur were observed (Remlinger, 1905). The original vaccine consisted of fixed rabies virus that has been grown in rabbit CNS tissue. An extremely small percentage of those who received the vaccine developed a monophasic paralysis termed acute disseminated encephalomyelitis (Stuart and Krikorian, 1928). Considering that paralysis is not a symptom associated with rabies infection and that individuals exposed to the vaccine but not infected with rabies virus also developed paralysis suggested that tissue from the CNS which contaminated the vaccine was responsible for the paralytic illness (Einstein et al., 1962). Initial studies showed that immunization with CNS tissue alone could induce demyelinating encephalomyelitis and that EAE could be induced in a wide range of species including mice, rats, guinea pigs, monkeys, sheep, dogs and chickens (Stuart and Krikorian, 1928; Paterson, 1976; Martonson, 1984).

Lymphocytes, as mediators of EAE, were first implicated by experiments in which anti-lymphocyte antibodies inhibited induction of EAE (Waksman et al., 1961). Further evidence that T cells were involved stemmed from the observation that thymocytes are required for EAE induction (Arnason et al., 1962). More specifically, the T helper subset appeared to be the primary candidate as shown by the elimination of Ly1 T cells preventing the transfer of EAE to naive recipients (Brostoff and Mason, 1984). Conformation of the involvement of CD4 T cells is their abundant presence in inflammatory EAE lesions in the CNS (Traugott et al., 1986).

As MBP is the primary autoantigen in EAE, the various epitopes of the protein have been the subject of intense study. Using peptic fragmentation and the synthetic peptide approach, epitopes of MBP that are encephalitogenic have been identified. Different species appear to respond to different regions of MBP. In the H-2^u mice, PL/J and B10.PL, the amino terminal region 1-37 can induce EAE while the H-2^s mice, SJL/J and A.SW, recognize MBP 89-169 as an encephalitogenic epitope (Fritz et al., 1983; Fritz et al., 1985). The encephalitogenic determinant in the Lewis rat strain has been shown to be MBP 68-88. Further characterization of these regions has been conducted and specific amino acid residues have been identified as being responsible for conferring encephalitogenicity (Zamvil et al., 1986). An intriguing discovery in the T cell subsets responsive to some epitopes of MBP demonstrated restricted TCR usage. The CD4 T cells in PL/J mice and Lewis rats that mediate EAE are Vß8.2 specific. Experiments employing anti-Vß8.2 antibodies have successfully prevented the induction of EAE (Acha-Orbea et al., 1988). Also, peptides of the Vß8.2 TCR were shown to be able to block induction of EAE in Lewis rats. Thus, insight into T cell responses to MBP has provided clues to the mechanism of such pathogenesis.

The EAE model has also served as a vital means for testing of novel forms of immunotherapy. Among the most recently tested and successful treatments are anti-TCR antibodies, anti-MHC antibodies, anti-CD4 antibodies, T cell vaccination and peptide therapy (Steinman et al., 1983; Brostoff and Mason, 1984; Howell et al., 1989; Vandenbark et al., 1989). Development of effective and safe forms of therapy in EAE is the first step toward testing potential therapies in human autoimmune disease.

The Interferons

The family of secretory proteins known as interferons (IFNs) were first described in 1957 by Issacs and Lindenmann (Issacs and Lindenmann, 1957). Their name is derived from their ability to interfere with virus replication. In addition to their antiviral effects, these molecules possess many varied activities including those which are antimicrobial, antitumor, and immunomodulatory (Pestka et al., 1987). Advances in the understanding of the basic mechanism of IFN actions and a number of clinical trials have led to Food and Drug Administration approval of IFN as treatment for several diseases including hairy cell leukemia, condyloma acuminatum, acquired immune deficiency syndrome (AIDS) related Kaposi's sarcoma, type C hepatitis, and most recently multiple sclerosis.

The IFN family of molecules are divided into three main species: IFNα, IFNß, and IFNγ. Two other types of IFNs, IFNτ and IFNω, are close relatives of IFNα. These molecules can be further classified into two distinct groups. The type I IFNs include IFNs α, ß, τ, and ω, and are induced primarily by viruses and tumor cells while type II (immune) IFN or IFNγ is induced by antigens and mitogens that stimulate T and NK cells. There are as many as 25 species of structurally similar forms of IFNα, encoded by a family of as many IFNα genes (Allen, 1982). A number of IFNα classes exist and recently the IFNα2 family was renamed IFNω. Unlike IFNα, IFNß is encoded by a single IFNß gene and the mature protein is composed of 166 amino acid residues. It was thought that an additional IFNß subtype had been identified. However, the protein possessed no antiviral activity and is presently referred to as interleukin-6 (Van Damme et al., 1987).

While the first described IFNs have been well studied, the type I IFN, IFNτ, has only been recently characterized. IFNτ was first identified as a major conceptus secretory protein in sheep, and in its reproductive function, it is important for its antileuteolytic properties (Godkin et al., 1982). IFNτ shares 45-55 % amino acid sequence homology with various IFNαs and is as potent as any IFNα in its antiviral activity (Imakawa et al., 1987; Pontzer et al., 1988).

IFNγ is a pleiotropic lymphokine with numerous unique biologic effects. IFNγ is predominantly made by T_{H1} cells and CD8 cytotoxic T lymphocytes (Salgame et al., 1991). IFNγ, while able to exert antiviral activity, is not as potent as the type I IFNs. Other biological activities of IFNγ include, macrophage activation, upregulation of MHC class I and II on macrophage and B cells, and B cell maturation (Pestka et al., 1987).

The suppressive and antiproliferative effects of the IFNs as applied to cellular growth and viral replication, coupled with their potent immunomodulatory effects render them as attractive agents for use in combating autoimmune disease. This is evidenced by the amelioration of the relapsing-remitting nature of multiple sclerosis by IFNß (IFNß Multiple Sclerosis Study Group, 1993). Furthermore, if superantigens do, as speculated, play a role in the initiation or persistence of autoimmune disease, then the suppressive effect of IFNs on superantigen-driven activation, presented in this dissertation, may form the basis of future therapies for autoimmune disease.

MATERIALS AND METHODS

Synthetic Peptides

Overlapping peptides corresponding to the entire sequence of both TSST-1 and SEB were synthesized on a Biosearch 9500AT automated peptide synthesizer (Milligen/Biosearch, Burlington, MA) using 9-fluoroenylmethyl oxycarbonyl (FMOC) chemistry (Chang and Meienhofer, 1978). N-Terminal truncations of SEB(179-212) were generated by removal of the peptidyl resin from the reaction vessel when the desired sequence length was obtained. Peptides were cleaved from the resins using trifluoroacetic acid/ethanedithiol/thioanisole/crystalline phenol/distilled water at a ratio of 80/3/5/7/5. The cleaved peptides were then extracted in ether and subsequently dissolved in water and lyophilized. Reverse phase high performance liquid chromatography (HPLC) (Perkin Elmer, Norwalk, CT) analysis of the crude peptides revealed one major peak in each profile. Amino acid analysis of the peptides, performed by the University of Florida Protein Core Facility, showed that the amino acid composition corresponded closely to theoretical values.

Cell Lines and Reagents

Three cell lines were employed for the structural binding studies. The Raji is an EBV-transformed human B cell line that bears DR3, DRw10, DQw1 and DQw2 (Merryman et al., 1989). The A20 line is a murine B cell line that bears I-A^d and I-E^d (Russell et al., 1990). Both the Raji and A20 lines were obtained from ATCC

Table III. Steps in peptide synthesis

Step	Effect
Step 1: Deprotection	Removal of Fmoc group
Step 2: Neutralization and activation	Conversion of α-amino group from protonated to deprotonated form
Step 3: Coupling	Coupling of amino acid to nascent peptide chain Multi-coupling if poor coupling efficiency is observed
Step 4: Capping	Permanent acylation of unreacted α-amino groups, elimination from further participation in synthesis

(Rockville, MD). DR1-transfected L cells were kindly provided by Dr. Eric O. Long (National Institutes of Health), and are described elsewhere (Long et al., 1991). The IL-2 dependent cell line, HT-2, was kindly provided by Dr. Janet Yamamoto (University of Florida, Gainesville, FL). SEB, TSST-1 and SEA were obtained from Toxin Technology (Sarasota, FL). Anti-Vß antibodies were obtained from T Cell Sciences (Cambridge, MA), AMAC (Westbrook, ME) and Pharmingen (San Diego, CA). Human IFNα (HuIFNα) and human IFNß (HuIFNß) were purchased from Lee Biomolecular (San Diego, CA). Bovine IFNτ (BoIFNτ) was kindly provided by Drs. Fuller Bazer and Troy Ott (Texas A&M University, College Station, TX). Human IFNγ (HuIFNγ) was purchased from Intergen (Purchase, NY).

Radioiodinations

SEB and TSST-1 (2.5 μg) were radioiodinated with 500 μCi of $Na^{125}I$ (15 mCi/μg, Amersham Corp., Arlington Heights, IL) in 25 μl of 0.5 M potassium phosphate buffer, pH 7.4, and 10 μl of chloramine-T (5 mg/ml) for 2 min. After neutralization of the reaction with 10 μl vol of sodium bisulfite (10 mg/ml), potassium iodide (70 mg/ml), bovine serum albumin (BSA) (20 mg/ml) and 15 μl of NaCl (4 M), the preparation was sieved on a 5 ml Sepharose G-10 column. The fraction containing the highest radioactivity in the first eluted peak was used in the radioligand binding assays. The specific activity of the iodinated proteins ranged from 40 to 100 μCi/μg.

Peptide Competition Studies

For analysis of TSST-1 peptides, 2 x 10^5 cells of Raji or A20 line were washed with phosphate buffered saline (PBS), and incubated with 50 μl of competitor, unlabeled toxin or PBS at room temperature for 1 hr. The cells were then incubated with ^{125}I-TSST-1 in PBS/1 % BSA for 1 hr. 75 μl of the mixture was placed on 0.65 μ filter units (Millipore, Bedford, MA), spun, washed twice with PBS/1 % BSA and counted on a gamma counter. For analysis of class II MHC peptides, 50 μl of various concentrations of peptide were preincubated with 50 μl of ^{125}I-TSST-1 at room temperature for 1 hr. Peptide and labeled TSST-1 were then incubated with 2 x 10^5 of either Raji or A20 cells at room temperature for 1 hr. Samples were then harvested and counted as described above.

For analysis of SEB peptides, DR1-transfected L cells were plated into a 96 well microtiter plate at a concentration of 6 X 10^4 cells per well and allowed to adhere and grow to confluency over 24 hrs at 37°C. Media was removed and 50 μl of competitor were added and incubated with the cells at room temperature for 1 hr. The cells were then incubated with ^{125}I-SEB in PBS/1 % BSA for 1 hr. The cells were washed twice with PBS/1 % BSA and solubilized by 1 % SDS. The liquid was then absorbed by cotton tip applicators and assayed on a gamma counter. For analysis of Raji cells, 2 x 10^5 cells of the Raji line were washed with PBS, and incubated with 50 μl of competitor, unlabeled toxin or PBS at room temperature for 1 hr. The cells were then incubated with ^{125}I-SEB in PBS / 1 % BSA for 1 hr. 75 μl of the mixture was placed on 0.65 μ filter units (Millipore, Bedford, MA), spun, washed twice with PBS / 1 % BSA and assayed on a gamma counter.

Induction of EAE

Six to eight week old female PL/J mice (The Jackson Laboratory, Bar Harbor, Maine) were immunized subcutaneously at the base of the tail with 300 μg rat MBP in an emulsion of equal volumes of complete Freund's adjuvant (CFA) containing H37Ra (4 mg/ml) and PBS. On the day of injection with antigen and 48 hrs later, *Bordetella pertussis* toxin (List Biological Laboratories, Campbell, CA) (400 ng) was injected i.p.. Mice were examined daily for signs of EAE. Mice that were treated with SEB to prevent EAE received 40 μg SEB in 0.2 ml PBS i.p. while untreated mice received 0.2 ml PBS and five days later were injected with rat MBP as described above.

Injection Schedule for Re-activation of EAE by Staphylococcal Enterotoxins

For injection of staphylococcal enterotoxin for initial re-activation of disease, SEB (40 μg) (Sigma Chemicals, St. Louis, MO), SEA (40 μg) (Toxin Technology, Sarasota, FL) in 0.2 ml PBS and pertussis toxin (500 ng) both were administered i.p. on the same day one month after resolution of clinical symptoms. For subsequent re-activations of EAE by SEB, 40 μg of SEB in 0.2 ml PBS was administered i.p. with or without pertussis toxin seven to nine days after resolution of clinical symptoms.

Flow Cytometry

For analysis of superantigen treated PL/J mice, spleens were removed from untreated and SEB treated PL/J mice, prepared as single cell suspensions and treated with Tris-buffered 0.16 M ammonium chloride. 1 x 10^6 cells were washed with FACS buffer (PBS containing 0.5 % bovine serum albumin and 10 mM sodium azide) and then incubated with biotinylated anti-Vß8 or anti-Vß6 antibodies (Pharmingen, San Diego, CA) for 30 min at 37°C. Cells were washed and incubated with streptavidin-phycoerythrin for 15 min at room temperature. Cells were then washed twice and incubated with FITC labeled anti-CD4 or anti-CD8 antibodies (Pharmingen, San Diego, CA) for 30 min at 37°C. Cells were washed again and analyzed on a FACScan (Becton-Dickenson, Mountain View, CA) as 10,000 events per sample.

For Vß analysis of type I IFN effects on superantigen activity, HPMC treated with SEB in the presence or absence of the type I IFNs for 72 hr were washed with FACS buffer and then incubated with FITC conjugated anti-Vß antibodies for 1 hr at 37°C. Cells were washed, resuspended in 1 ml FACS buffer and analyzed on a FACScan (Becton-Dickinson, Mountain View, CA) in duplicate as 10,000 events per sample.

Proliferation Assays

For SEB peptide studies, human peripheral mononuclear cells (HPMC) were separated from heparinized blood donated by healthy volunteers using Histopaque density centrifugation. HPMC were collected, washed and resuspended in RPMI 1640 medium supplemented with 5 % fetal bovine serum, 0.1 mM 2-mercaptoethanol, and antibiotics (100 units of penicillin, 100 μg of streptomycin per ml). Cells (5 x 10^5) were added in 96-well microtiter plates and preincubated with the various SEB peptides in triplicate at 37°C for 3 to 5 hr. SEB was added at a final concentration of 10 pg/ml and the cells were incubated for 3 days. The cells were pulsed with 1 μCi of [^{3}H]thymidine for 18 hr before harvesting. Cells were harvested on a model M12 Brandel cell harvester (Gaithersburg, MD) and [^{3}H]thymidine incorporation was determined in a ß-scintillation counter.

For EAE prevention studies, spleen cells from either SEB treated or untreated EAE resolved PL/J mice were ammonium chloride treated and stimulated in vitro with SEB or SEA in 96-multiwell tissue culture plates at 5 x 10^4 cells per well. Incubation and harvest were performed as stated above.

For EAE re-activation studies, spleen cells were obtained from PL/J mice seven days after last injection or immunization and the red cells lysed with 0.84 % ammonium chloride. Spleen cells (3 x 10^5/well) were incubated for 3 days in round bottom microtiter wells that had been coated with an anti-Vβ8 antibody. The purified anti-V$\beta8^+$ antibody, F23.1, was diluted to 10 μg/ml with PBS and 30 μl added per microtiter well. Plates were incubated at 37°C for 2 hours and washed with PBS before adding lymphocytes. Incubation and harvest was performed as stated above.

For IFN and superantigen studies, HPMC (2.5 x 10^5/well) were added to 96-well microtiter plates and incubated with the various superantigens and IFNs in quadruplicate at 37°C. Incubation and harvest were performed as stated above.

IL-2 Bioassay

IL-2 activity of 48 hr supernatants was determined using the IL-2 dependent cell line, HT-2 (Ho et al., 1987). HT-2 cells (10^4/well) were added in a 96-well microtiter plate and incubated with supernatants and serial dilutions of recombinant human IL-2 (rHuIL-2) for the generation of a standard curve. Plates were incubated for 72 hr and pulsed with 1 μCi of [^{3}H]thymidine for 6 hr before harvesting. Cells were harvested on a model M12 Brandel cell harvester (Gaithersburg, MD) and [^{3}H]thymidine incorporation was determined in a ß-scintillation counter.

Circular Dichroism (CD)

CD for peptides was determined at room temperature using a JASCO 500C spectropolarimeter. Scans were done with a 0.1 mm path length cell at a sensitivity of 2.0 and a time constant of 8 sec. The wavelength range measured from 250 nm to 188 nm at a scan rate of 10 nm/min. Scans were carried out on peptides in water at concentrations of 0.1-0.5 mg/ml. The CD spectra were the average of three scans and expressed in terms of ϵ (Yang et al 1986):

$$\epsilon = [\theta] /3298 \; ; \; [\theta] = [\theta]_{observed} / c \times l$$

where $[\theta]$ and $[\theta]_{observed}$ are expressed in degrees, c equals the mean residue concentration in mol/liter, and l is the path length of the cell in cm. Secondary structure was estimated with the SSE program provided by JASCO Inc.

Statistical Analysis

Statistical analysis of experiments involving mouse treatment groups was performed using a nested design with mice nested with in treatment groups. Mice were considered a random effect. Statistical significance of certain data presented in this dissertation (at alpha level 0.05) was assessed by analysis of variance followed by Student's T-test and ANOVA. All statistical analysis was done under consultation with the University of Florida Statistics Consulting Division.

RESULTS

TSST-1 Peptide Binding Studies

Overlapping peptides of the entire TSST-1 molecule were synthesized and tested for their ability to block binding of TSST-1 to the human Burkitt's lymphoma line, Raji and the murine B cell line, A20. Peptide sequences and secondary structure as predicted by CD are presented in Table IV. Peptides were designed based upon the composite surface profile and the primary sequence of TSST-1. The predicted composite surface profile of TSST-1 is presented in Figure 4. The CD of the TSST-1 peptides revealed a predominance of β-structure relative to α-helix. It has been shown that TSST-1 binds to HLA-DR, HLA-DQ but with low affinity to HLA-DP (Scholl et al., 1990). The Raji line bears DR3 and DRw10 as well as DQw1 and DQw2 and the A20 line bears I-A^d and I-E^d (Merryman et al., 1989). Competitive binding studies between TSST-1 peptides and ^{125}I-TSST-1 molecule for MHC class II antigens were performed to determine the region on TSST-1 involved in interaction with the class II MHC molecule. Of the seven peptides tested, T(39-68) and T(155-194) were able to displace binding of ^{125}I-TSST-1 to Raji and A20 cells, suggesting that these regions on TSST-1 are involved in binding to the class II molecule (Figure 5). The peptide, interferon γ(108-133), was tested as a negative control, and did not compete with TSST-1. Dose response studies were performed on Raji cells to determine the relative blocking ability of the peptides with activity in Figure 5. 50 % inhibition of binding was observed for T(39-68) at 80 μM and for T(155-194) at 30 μM (Figure 6). In addition, a scrambled peptide of T(39-68) (Table IV), the sequence of which was produced by the sequence edit program (Devereux et al., 1984), was tested to determine if the binding of T(39-68) was sequence specific. T(39-68)S did not block ^{125}I-TSST-1 binding at 500 μM indicating that binding of T(39-68) was sequence specific. A scrambled peptide of T(155-194) was also unable to inhibit binding of ^{125}I-TSST-1 (data not shown). Similar dose dependent patterns of inhibition for the peptides with inhibitive activity and their nonactive scrambled counterparts was observed on murine A20 cells (data not shown). Thus, the N-terminal peptide T(39-68) and the C-terminal peptide T(155-194) represent regions of TSST-1 that are involved in binding to both HLA and MHC class II molecules on Raji and A20 cells.

In order to determine the region of the class II molecule that binds TSST-1, class II MHC, I-A^b, ß-chain peptides I-A$_ß{}^b$(30-60), I-A$_ß{}^b$(60-90), I-A$_ß{}^b$(65-85), and IA$_ß{}^b$(80-100) were examined for their ability to inhibit binding of TSST-1 to Raji and A20 cells (Russell et al., 1990). Peptides I-A$_ß{}^b$(30-60) and I-A$_ß{}^b$(60-90) preferentially inhibited binding of ^{125}I-TSST-1 in a dose dependent manner to both Raji cells (Figure 7) and A20 cells (data not shown) The peptide I-A$_ß{}^b$(60-90) encompasses the entire ß-chain α-helix while I-A$_ß{}^b$(30-60) is a beta-turn underlying the N-terminal region of the (60-90) α-helix (Bjorkman et al., 1987; Brown et al., 1988). Thus, TSST-1 interacts with residues of the α-helix and ß-turn of the class II MHC molecule ß-chain.

Table IV. Amino acid sequences and secondary structure of TSST-1 peptides as determined by CD

TSST-1 peptides	Sequences	% α helix	% ß sheet
T(1-45)	STNDNIKDLLDWYSSGSDTFTNSEVLDNSLGSMRIKNTDGSISLI	8	29
T(39-68)	DGSISLIIFPSPYYSPAFTKGEKVDLNTKR	9	21
T(63-94)	DLNTKRTKKSQHTSEGTYIHFQISGVTNTEK	7	29
T(89-122)	TNTEKLPTPIELPLKVKVHGKDSPLKYGPKFDKK	3	40
T(118-140)	KFDKKQLAISTLDFEIRHQLTQI	7	22
T(133-159)	IRHQLTQIHGLYRSSDKTGGYWKITMN	10	62
T(155-194)	KITMNDGSTYQSDLSKKFEYNTEKPPINIDEIKTIEAEIN	7	24
T(39-68)S†	TVNGIPRDYLDSYSTPGKSIFKKEILPSFA	-	-
T(155-194)S†	EDFEKPNINEKNDLTKTADIYGEMINIYEKTKSQITISPS	-	-

Note: Secondary structure estimates were done using the SSE program provided by JASCO Inc.

†S denotes a scrambled version of the original sequence (Devereux et al., 1984).

Figure 4. Predicted composite surface profile for TSST-1 using the three parameters of HPLC hydrophilicity, accessibility, and segmental mobility (Parker et al., 1986). Residues with composite profile values greater than 50 % are predicted to be more likely to be accessible to the surface of the molecule.

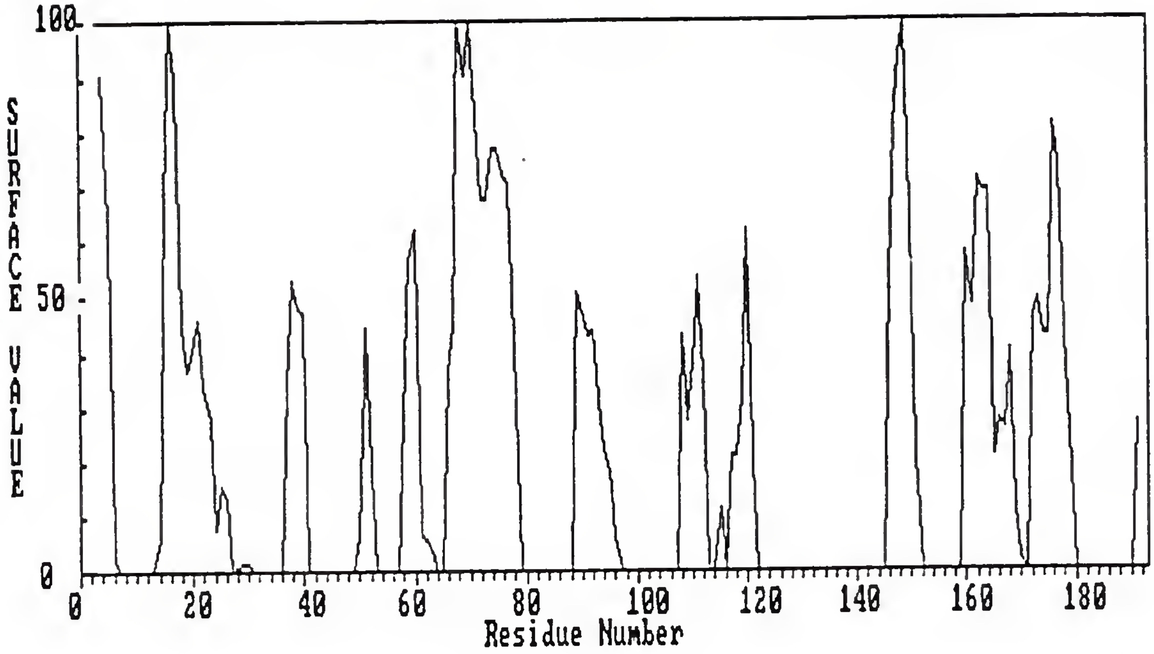

SURFACE VALUE
100
50
0
0
20
40
60
80
100
120
140
160
180
Residue Number

Figure 5. Percent control binding of ^{125}I-TSST-1 to Raji and A20 cells in the presence of TSST-1 peptides. TSST-1 peptides were used at a final concentration of 500 μM. Iodinated TSST-1 was used at a final concentration of 2.5 nM. IFNγ(108-133) was used at a final concentration of 500 μM for control. Each bar represents the mean percent of TSST-1 control binding in the presence of TSST-1 peptides ± SE.

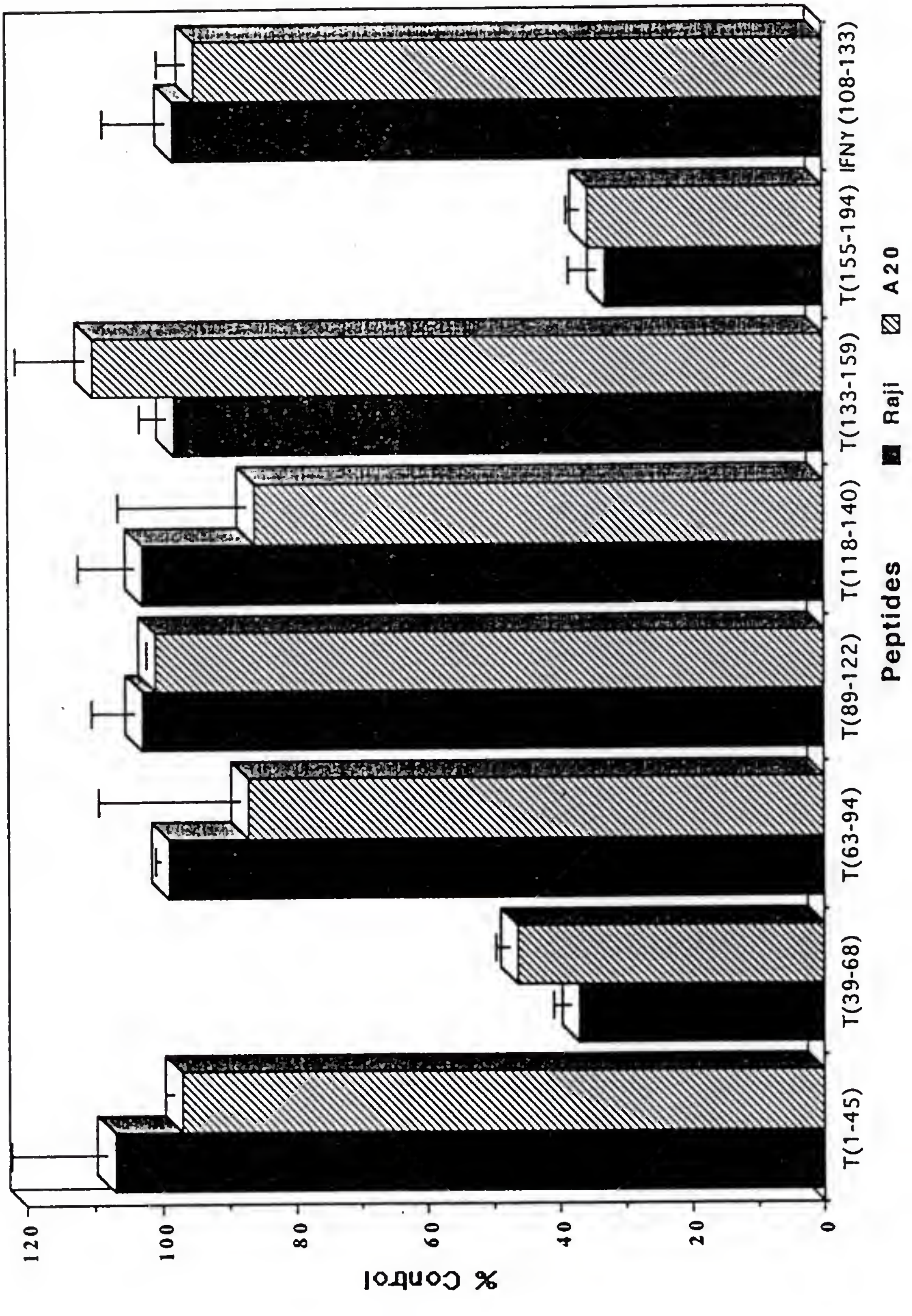

% Control
0
20
40
60
80
100
120
T(1-45)
T(39-68)
T(63-94)
T(89-122)
T(118-140)
T(133-159)
T(155-194)
IFNγ (108-133)
Peptides
■ Raji
▨ A20

Figure 6. Dose dependent inhibition of ^{125}I-TSST-1 binding to Raji cells in the presence of T(39-68), T(155-194) and T(39-68)S. T(39-68)S is a scrambled peptide whose sequence was generated via the sequence edit program (Deveraux et al., 1984)). Labeled TSST-1 was used at a final concentration of 2.5 nM. In the absence of competitor, binding of ^{125}I-TSST-1 to Raji cells was 6407 ± 21 cpm. Each point represents the mean percent reduction ± SE of TSST-1 control binding in the presence of TSST-1 peptides.

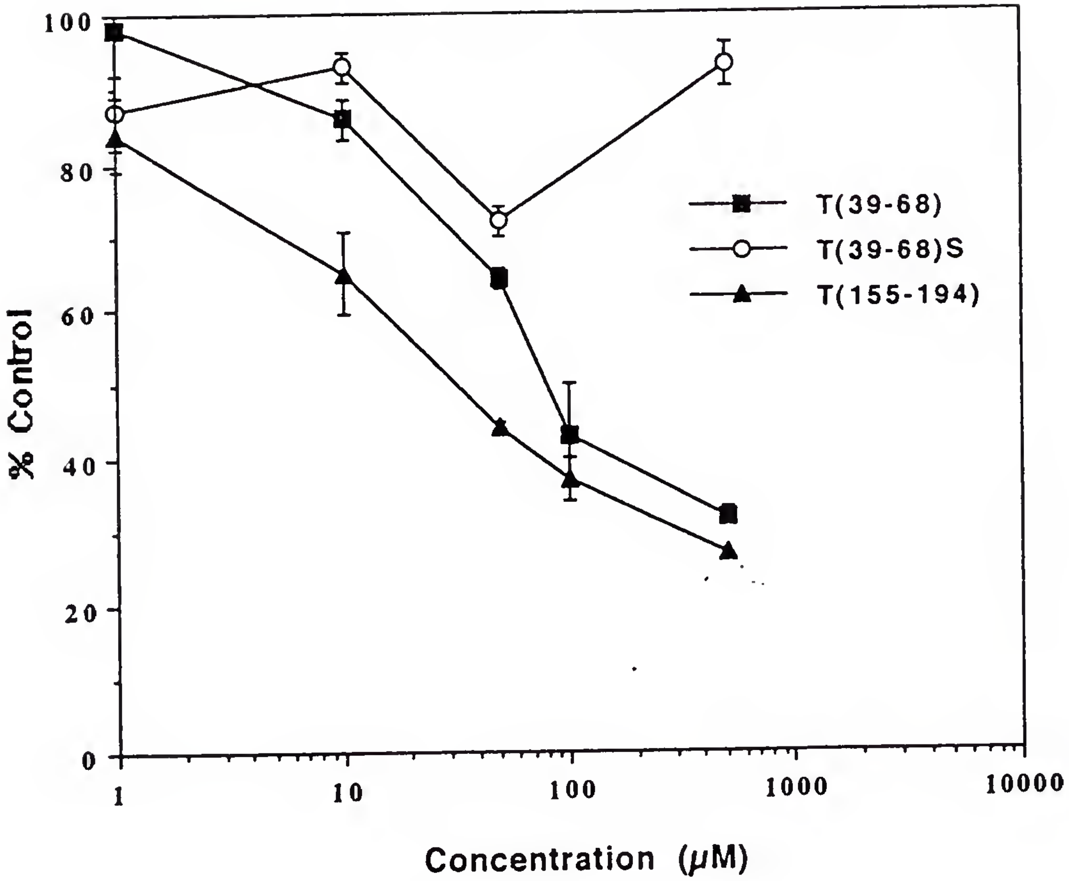
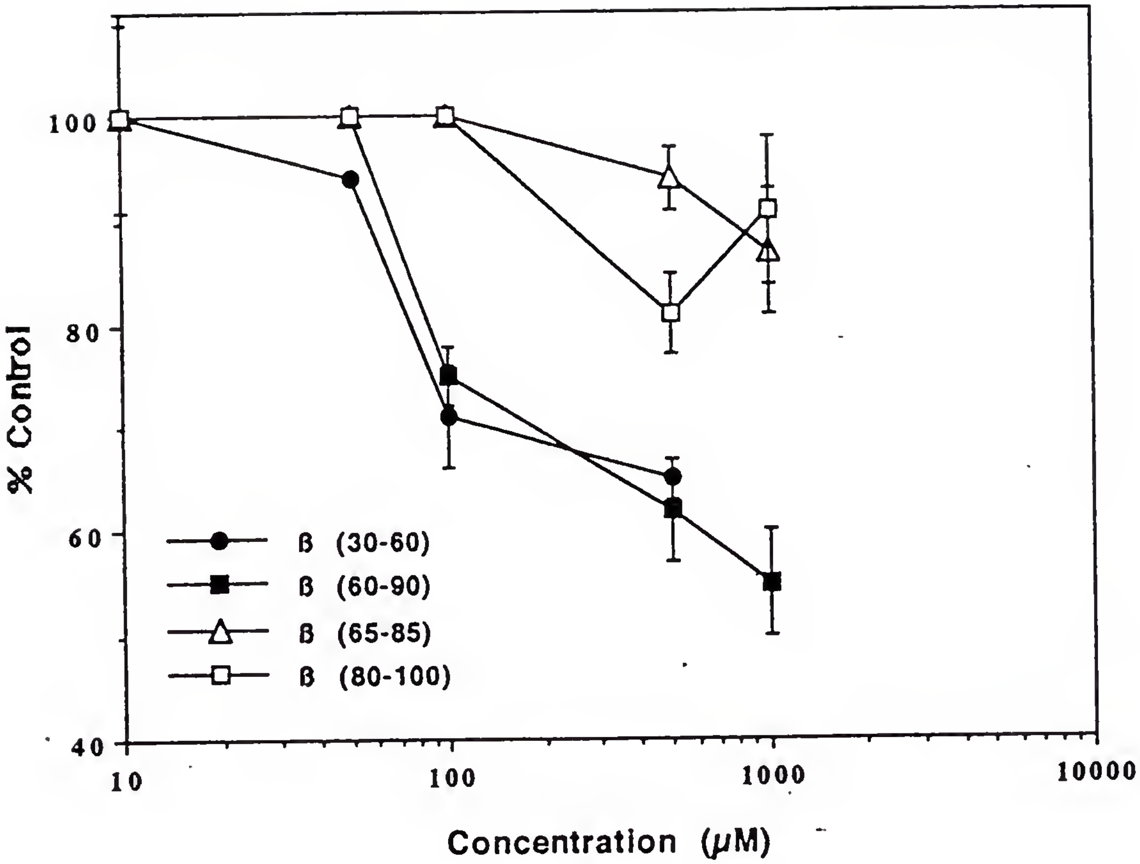

100
80
60
40
20
0
% Control
1
10
100
1000
10000
Concentration (μM)
T(39-68)
T(39-68)S
T(155-194)

Figure 7. Dose dependent inhibition of TSST-1 binding to Raji cells in the presence of ß-chain class II MHC peptides. ^{125}I-TSST-1 was used at a final concentration of 2.5 nM. Each point represents the mean percent reduction ± SE of TSST-1 control binding in the presence of class II MHC peptides.


100
80
60
40
% Control
10
100
1000
10000
Concentration (μM)
ß (30-60)
ß (60-90)
ß (65-85)
ß (80-100)

SEB Peptide Binding Studies

Overlapping peptides of the entire SEB molecule were synthesized and examined for their ability to inhibit binding of ^{125}I-SEB to DR1 transfected L cells. The peptides were designed and synthesized to correspond to discrete secondary structures of SEB and the sequences and corresponding regions they encompass in the crystal structure of SEB (Swaminathan et al., 1992) are presented in Table V. The predicted composite surface profile of SEB was also considered in the designation of peptide sequences (Figure 8). Competitive binding studies between SEB peptides and ^{125}I-SEB for MHC class II antigens were performed to determine the region(s) of SEB involved in interaction with the MHC class II molecule. Of the overlapping peptides tested, peptides corresponding to amino acid residues 1-33, 31-64 and 179-212 were able to displace binding of ^{125}I-SEB to DR1 transfected L cells (Figure 9). No appreciable binding of ^{125}I-SEB to L cells transfected with the DR α-chain, but which did not express surface DR1, was observed (data not shown). Dose response studies were performed on DR1 transfected L cells to determine the relative blocking abilities of the peptides possessing inhibitory activity in Figure 9. Each of the three peptides, (1-33), (31-64), and (179-212), showed inhibition of ^{125}I-SEB binding at concentrations as low as 20 μM, and caused 50 % inhibition of binding in the 100 μM range (Figure 10). In addition, IFNγ (108-133), an irrelevant peptide, was employed as a negative control and was shown not to inhibit ^{125}I-SEB binding. Thus, the amino terminal regions 1-33 and 31-64 and the carboxy terminal region 179-212 of SEB are involved in binding of SEB to the HLA-DR1 class II molecule.

We next determined the ability of the SEB peptides to inhibit binding of ^{125}I-SEB to the human Burkitt's lymphoma line, Raji. As stated previously, the Raji line bears HLA-DR3, DRw10, DQw1 and DQw2 (Merryman et al., 1989). The purpose of examining the activity of the SEB peptides against a second cell line bearing a different haplotype was to determine if the same or different regions of SEB were involved in binding to MHC class II molecules of different haplotypes. Of the eight SEB peptides tested, peptides corresponding to amino acid residues 1-33, 124-154, 150-183 and 179-212 were able to displace binding of ^{125}I-SEB to Raji cells (Figure 11). Dose response studies showed inhibition of binding at concentrations as low as 5 to 10 μM (Figure 12). 50 % inhibition of binding was observed for all four peptides in the range of 20 to 40 μM (Figure 12). IFNγ (108-133), an unrelated peptide, was also examined and did not compete with ^{125}I-SEB for binding. Thus, the regions of SEB encompassing residues 1-33, 124-154, 150-183 and 179-212 represent sites that are involved in binding to the class II molecules, DR3, DRw10, DQw1 and DQw2 on Raji cells. Further, while some SEB peptides inhibited SEB binding to both L cells transfected with DR 1 and Raji cells expressing different DR haplotypes, binding of SEB to the two cell lines was also differentially inhibited by distinct peptides. This suggests that different regions of SEB bind to different MHC class II molecule haplotypes.

Peptide (179-212) was selected to determine if peptide truncations could further define discrete regions or residues of peptides that are involved in binding

Table V. Amino acid sequences of and regions encompassed by SEB peptides.

Peptides	Sequences	Regions Encompassed
SEB 1 (1-33)	ESQPDPKPDELHKSSKFTGLMENMKVLYDDNHV	α1 & α2
SEB 2 (31-64)	NHVSAINVKSIDQFLYFDLIYSIKDTKLGNYDNV	β1 & β2
SEB 3 (60-94)	NYDNVRVEFKNRDLADKYKDKYVDVFGANYYYQCY	β3, α3 & β4
SEB 4 (90-128)	YYQCYFSKKTNDINSHQTDLRKTCMYGGVTEHNGNQLDK	β5
SEB 5 (124-154)	NQLDKYRSITVRVFEDGKNLLSFDVQTNKKK	β6 & β7
SEB 6 (150-183)	TNKKKVTAQELDYLTRHYLVKNKKLYEFNNSPYE	β8 & α4
SEB 7 (179-212)	NSPYETGYIKFIENENSFWYDMMPAPGDKFDQSK	β9 & β10
SEB 8 (208-239)	FDQSKYLMMYNDNKMVDSKDVKIEVYLTTKKK	α5, β11 & β12

Note: Peptide sequences were specifically designed to encompass the regions of SEB as assigned by the crystalline structure (Swaminathan et al., 1992).

Figure 8. Predicted composite surface profile for SEB using the three parameters of HPLC hydrophilicity, accessibility, and segmental mobility (Parker et al., 1986). Residues with composite profile values greater than 50 % are predicted to be more likely to be accessible to the surface of the molecule.

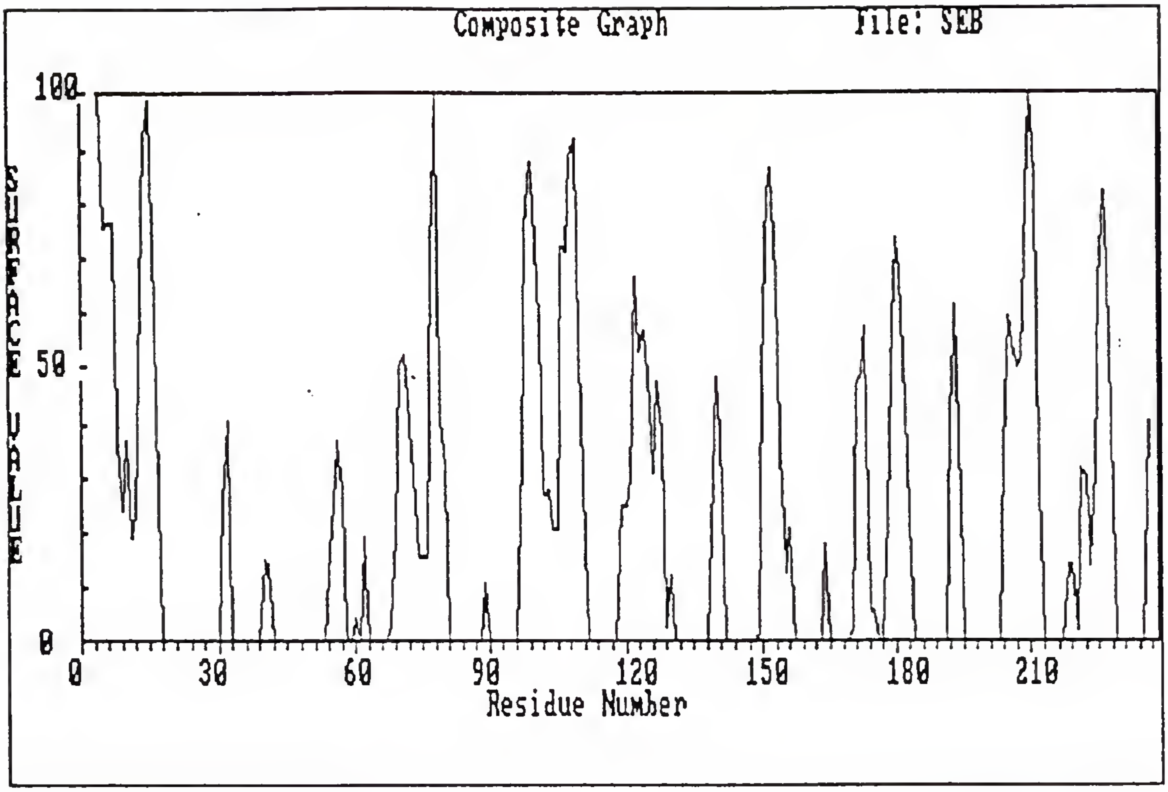

Composite Graph
File: SEB
SURFACE VALUE
100
50
0
0
30
60
90
120
150
180
210
Residue Number

Figure 9. Percent control binding of ^{125}I-SEB to DR1 transfected L cells in the presence of SEB peptides. SEB peptides were used at a final concentration of 250 μM. ^{125}I-SEB was used at a final concentration of 4 nM. Each bar represents the mean percent of SEB control binding in the presence of SEB peptides ± SE.

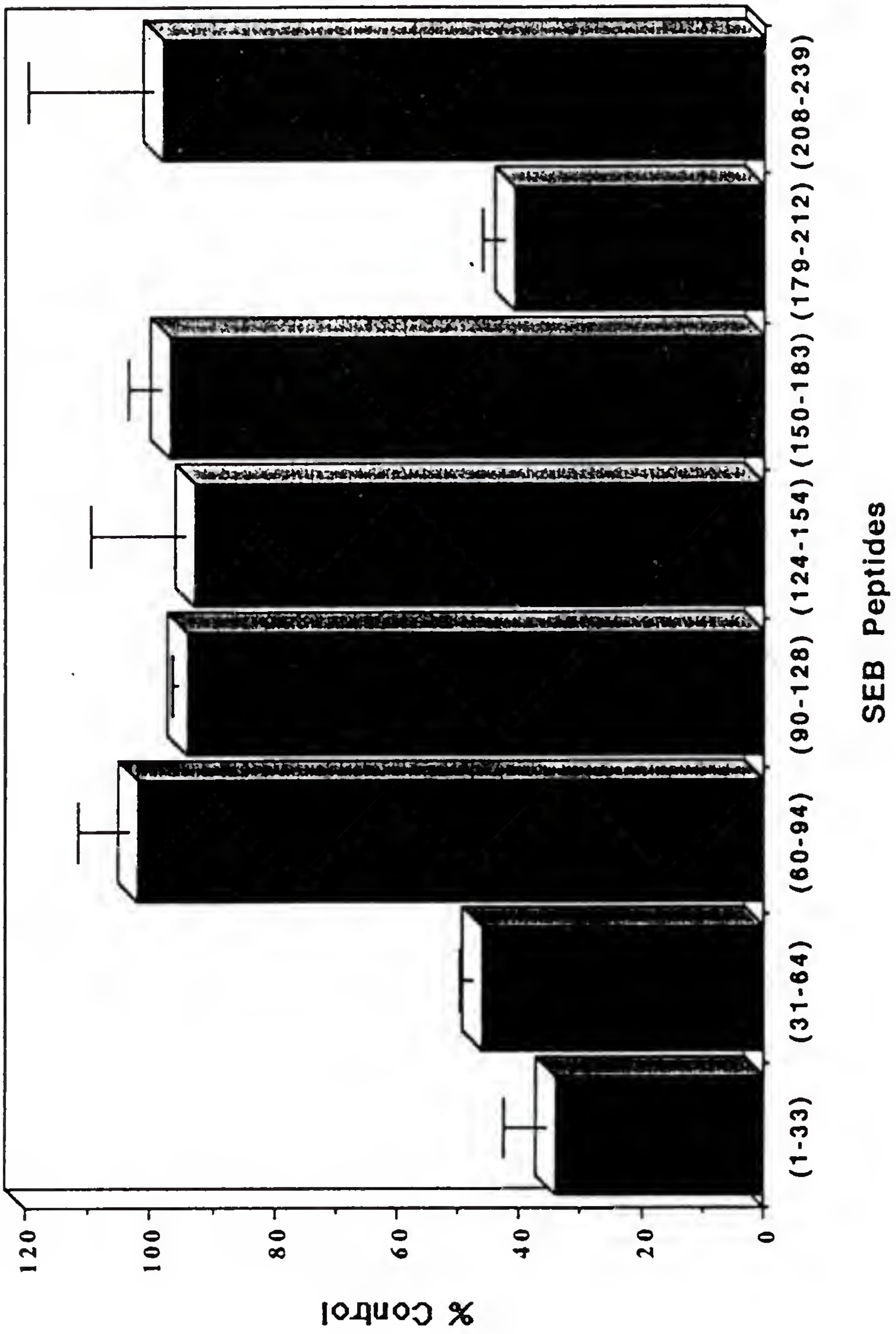

% Control
120
100
80
60
40
20
0
(1-33)
(31-64)
(60-94)
(90-128)
(124-154)
(150-183)
(179-212)
(208-239)
SEB Peptides

Figure 10. Dose dependent inhibition of ^{125}I-SEB binding to DR1 cells in the presence of SEB peptides possessing inhibitory activity. ^{125}I-SEB was used at a final concentration of 4 nM. Binding of ^{125}I-SEB in the absence of competitor was 2435 ± 187 cpm. Closed square (1-33), closed circle (31-64), closed triangle (179-212), and open triangle IFNγ (108-133). Each point represents the mean percent control ± SE.

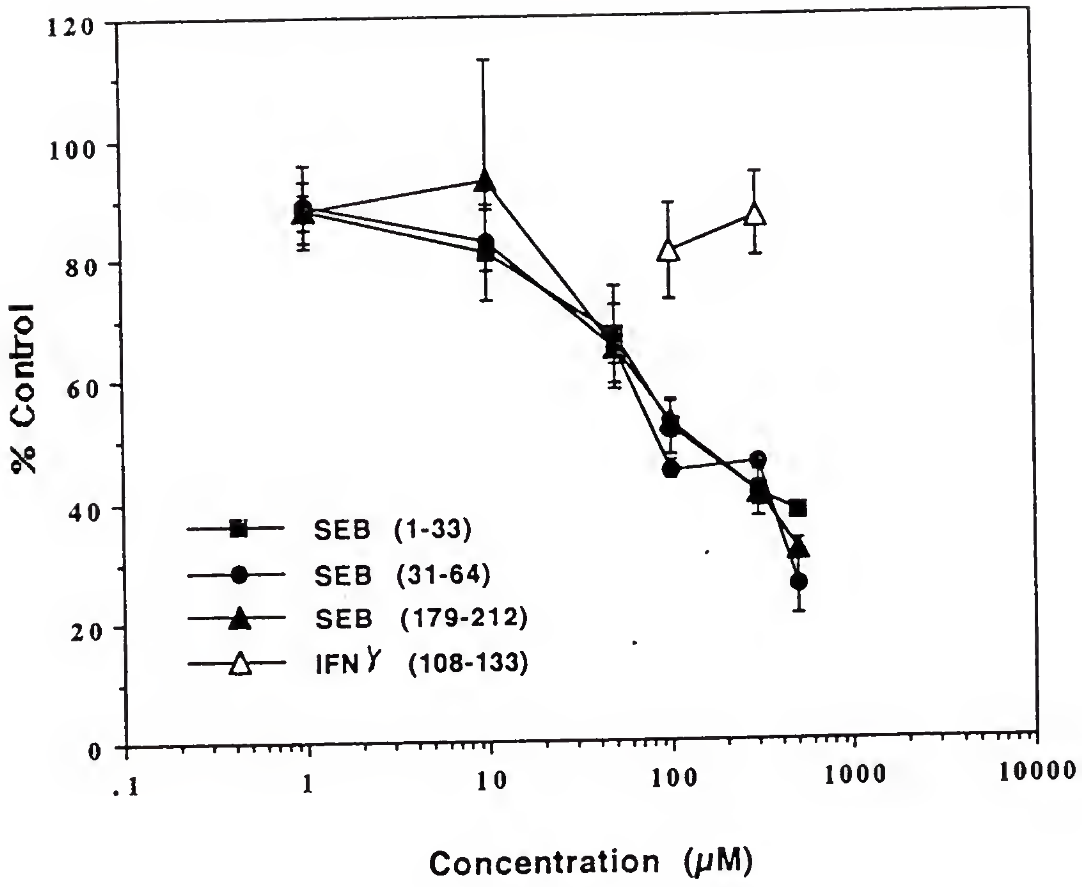

120
100
80
60
40
20
0
% Control
.1
1
10
100
1000
10000
Concentration (μM)
SEB (1-33)
SEB (31-64)
SEB (179-212)
IFNγ (108-133)

Figure 11. Percent control binding of ^{125}I-SEB to Raji cells in the presence of SEB peptides. SEB peptides were assayed at a final concentration of 300 μM. ^{125}I-SEB was used at a final concentration of 4 nM. Each bar represents the mean percent of SEB control binding in the presence of SEB peptides ± SE.

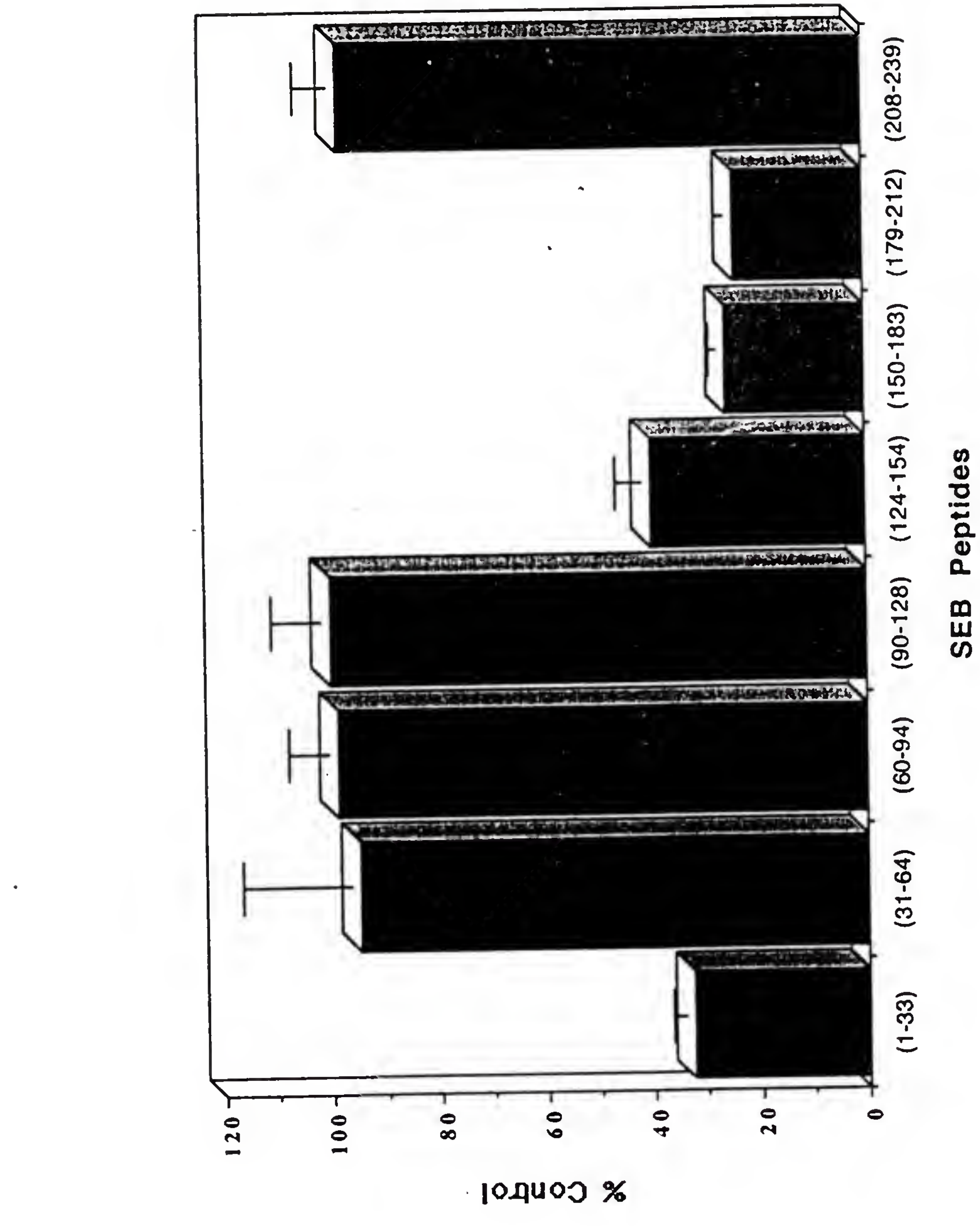

% Control
120
100
80
60
40
20
0
(1-33)
(31-64)
(60-94)
(90-128)
(124-154)
(150-183)
(179-212)
(208-239)
SEB Peptides

Figure 12. Dose dependent inhibition of ^{125}I-SEB binding to Raji cells in the presence of SEB peptides possessing inhibitory activity. ^{125}I-SEB was used at a final concentration of 4 nM. Binding of ^{125}I-SEB in the absence of competitor was 4768 ± 355. Closed square (1-33), closed circle (124-154), open square (150-183), closed triangle (179-212), and open triangle IFNγ (108-133). Each point represents the mean percent control ± SE.

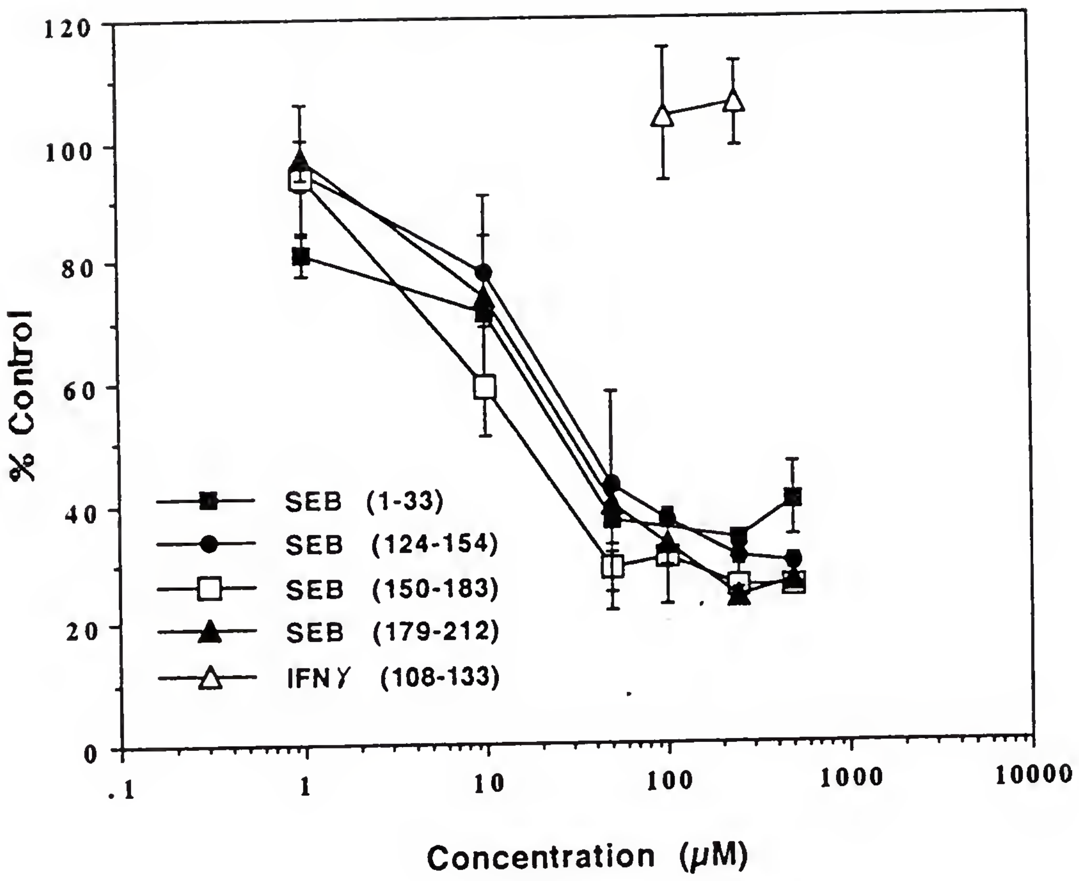
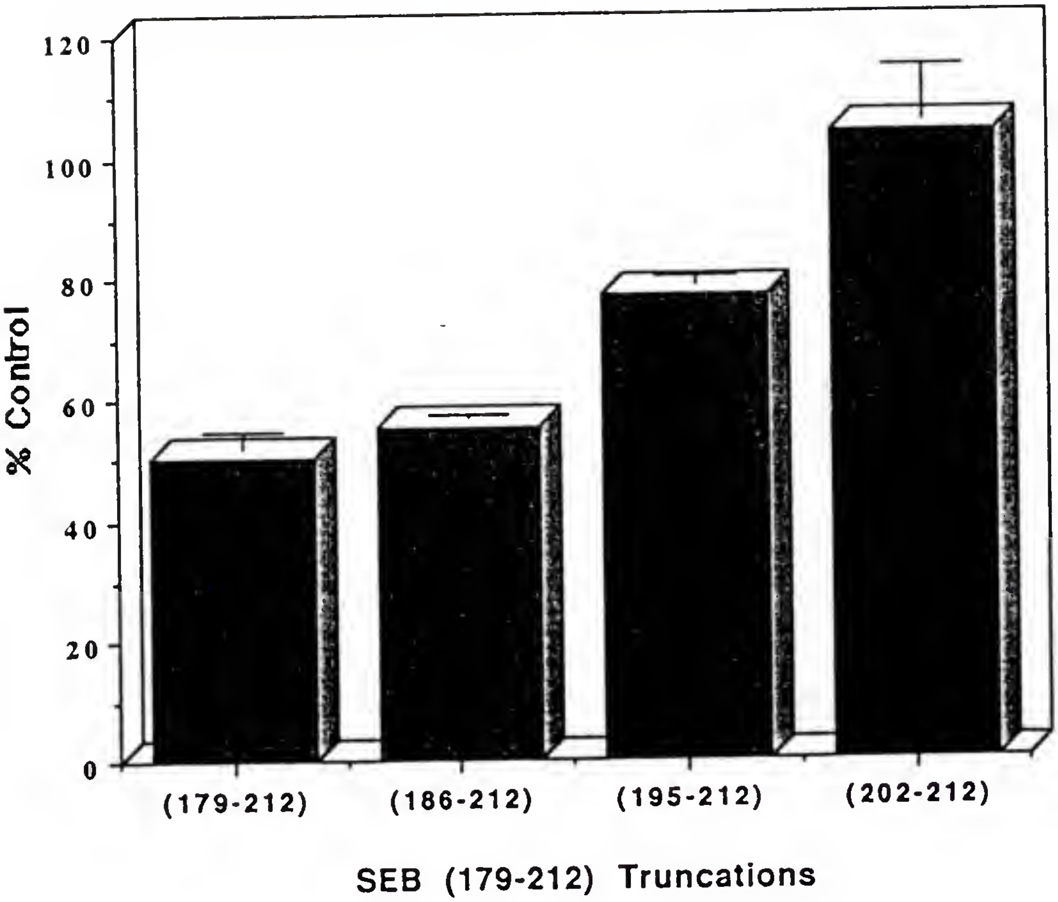

120
100
80
60
40
20
0
% Control
.1
1
10
100
1000
10000
Concentration (μM)
SEB (1-33)
SEB (124-154)
SEB (150-183)
SEB (179-212)
IFNγ (108-133)

Figure 13. Percent control binding of ^{125}I-SEB to DR 1 transfected L cells in the presence of N-terminal truncations of the SEB peptide (179-212). SEB peptide truncations were assayed at a final concentration of 200 μM. ^{125}I-SEB was used at a final concentration of 5 nM. Each bar represents the mean percent of SEB control binding in the presence of SEB (179-212) truncations ± SE.



% Control
120
100
80
60
40
20
0
(179-212)
(186-212)
(195-212)
(202-212)
SEB (179-212) Truncations

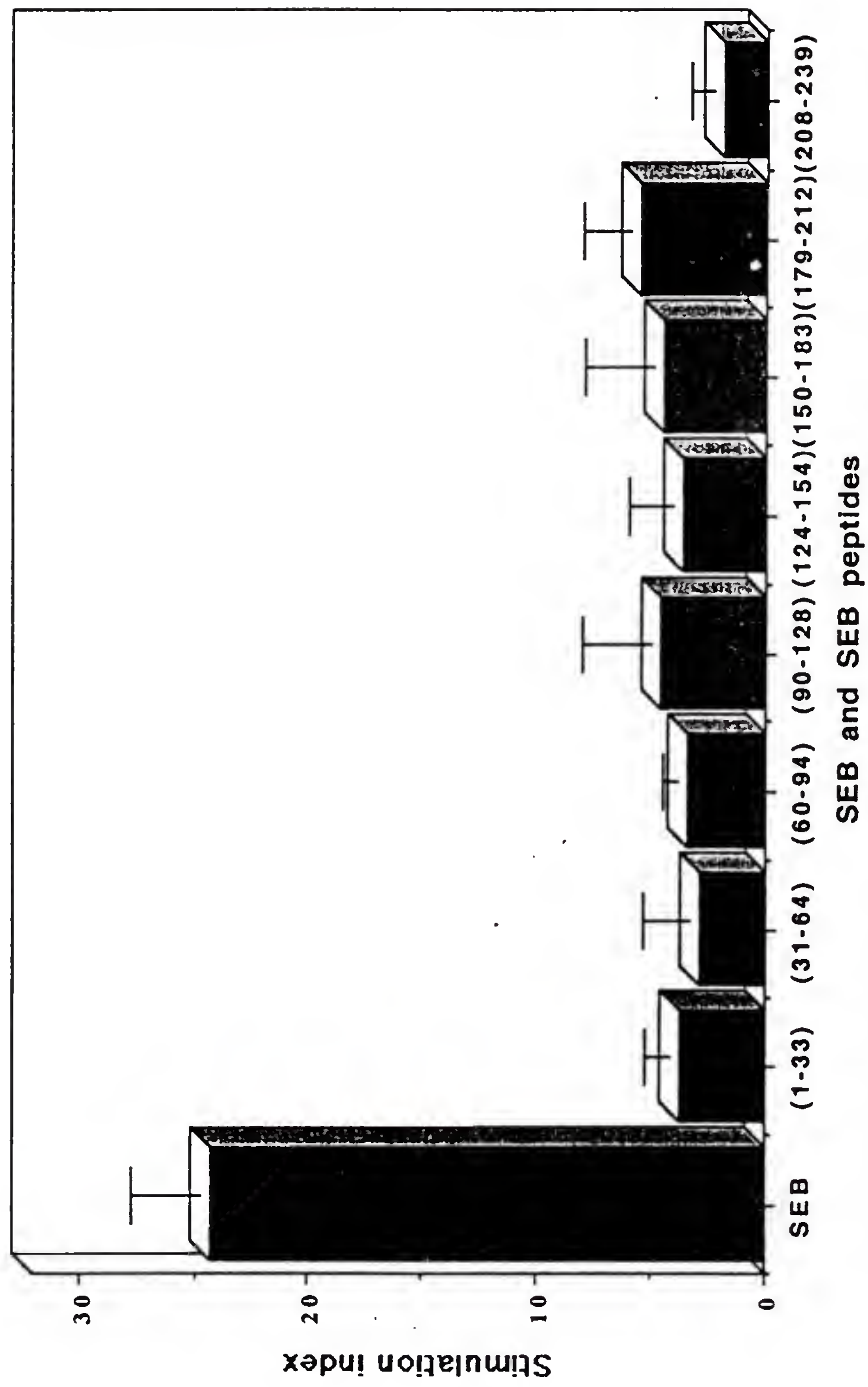
of SEB to HLA-DR1 molecules. Accordingly, we synthesized three amino-terminal truncations of SEB (179-212) encompassing regions 186-212, 195-212 and 202-212 as shown in Table VI. Competitive binding studies between the SEB (179-212) truncations and ^{125}I-SEB for DR 1 present on transfected L cells were performed. A gradual decline of competition was observed corresponding to a decrease in the size of the peptide (Figure 13). Similar results were observed with competitive binding studies conducted with Raji cells (data not shown). Such results suggest that the binding of the SEB peptide encompassing residues 179 thru 212 may not be localized to a specific region or residue or the truncations may have resulted in a corresponding reduction in the overall affinity and/or avidity of the peptide for binding to the MHC class II molecule.

Antagonist Activity of SEB(124-154)

Recent studies have suggested that peptides of the bacterial superantigen, SEA, have actual superantigenic properties themselves. The SEA peptide (121-149) is able to stimulate proliferation and cytokine induction (Pontzer et al., 1993), while SEA peptide (39-68) is able to stimulate proliferation (N. Griggs, personal communication). We tested the eight overlapping SEB peptides for their ability to induce T cell proliferation and found none of them capable of T cell stimulation (Figure 14). Another member of Dr. Johnson's laboratory, Dr. Carol H. Pontzer, tested the peptides for induction of TNF-α and β, however the studies were inconclusive.

We next attempted to determine if any of the SEB peptides were able to

Figure 14. The SEB peptides lack agonist activity. Data are expressed as stimulation indices ± SEM. HPMC (5×10^5 cells/well) were incubated with peptides at a final concentration of 300 μM or SEB at a final concentration of 0.1 μg/ml for 72 hr. Other peptide concentrations were employed and similar results were obtained. Peptides were shown to be noncytotoxic by trypan blue exclusion.

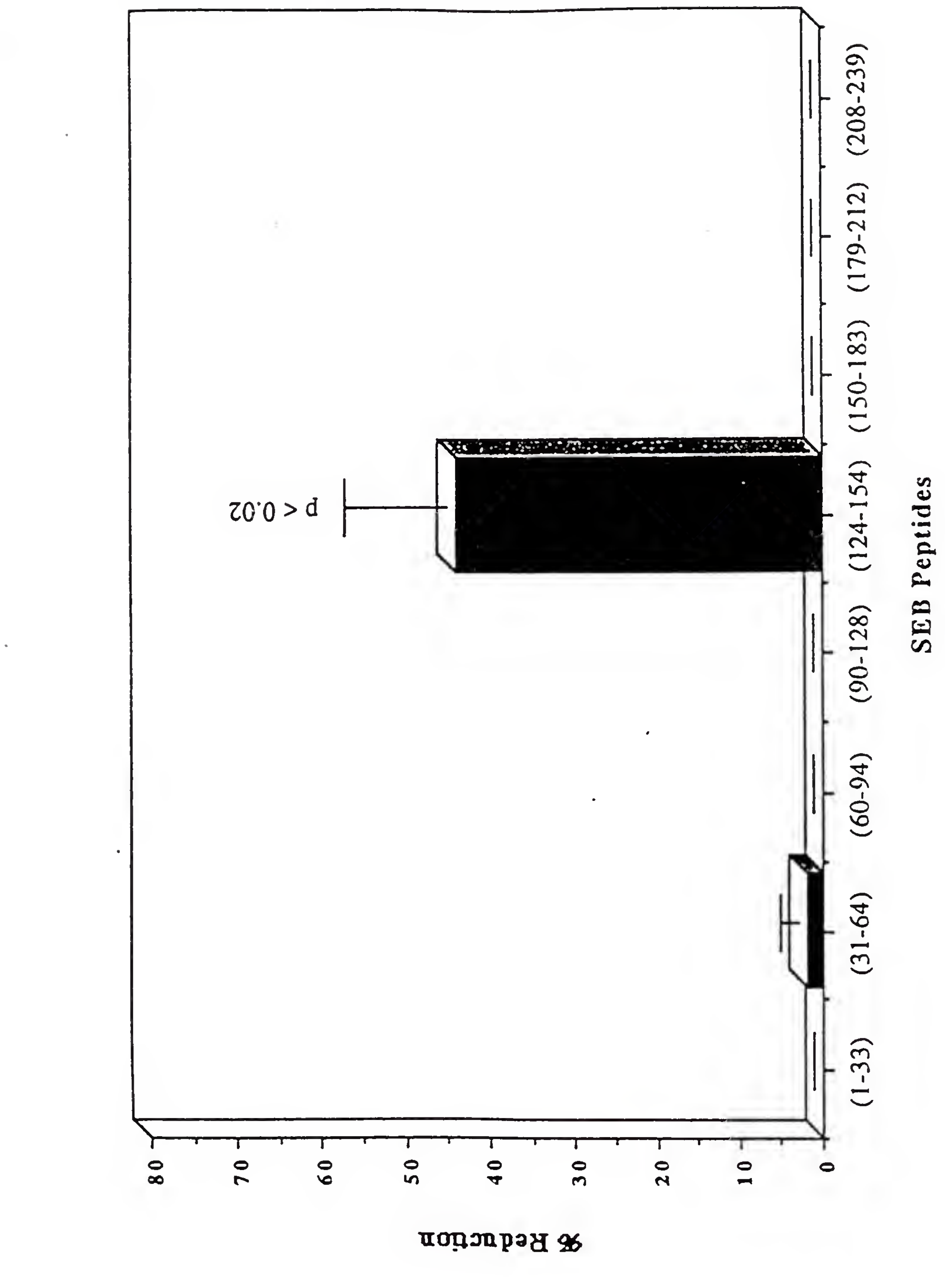

Stimulation index
0
10
20
30
SEB
(1-33)
(31-64)
(60-94)
(90-128)
(124-154)
(150-183)
(179-212)
(208-239)
SEB and SEB peptides

inhibit the mitogenic function of SEB, the eight peptides were tested for direct functional competition with SEB for inhibition of SEB-induced proliferation of HPMC (Figure 15). It was observed that at a concentration of 300 μM the peptide corresponding to amino acid residues 124-154 was able to significantly ($p < 0.02$) inhibit SEB-induced proliferation. The addition of an irrelevant peptide, IFNγ(108-133), had no effect on SEB-induced proliferation. These data suggest that residues 124 through 154 are part of a functional site directly responsible for activation of some lymphocytes by SEB. The other SEB receptor-binding sites may facilitate the direct activation of cells by residues encompassed in the 124-154 peptide.

Prevention of EAE by SEB

SEB has been shown to induce clonal anergy and deletion in peripheral Vβ8$^+$ CD4$^+$ T cells after in vivo administration (Kawabe and Ochi, 1990; Rellahan et al., 1990; Kawabe and Ochi, 1991). Interestingly, the culprit T cells in the PL/J mouse strain responsible for induction of EAE are Vβ8 specific (Acha-Orbea et al., 1988). In order to determine if mice treated with SEB could be protected from development of EAE, PL/J mice were injected i.p. with SEB (40 μg in 0.2 ml of PBS) or 0.2 ml PBS alone and allowed to rest for five days. SEB injected mice did not exhibit signs of toxicity at any time after administration.. After a five day period, treated mice and control PBS injected mice were immunized with rat MBP as described in Materials and Methods and observed for signs of disease. In Table VI, Experiment 1, five out of six mice in the group that received rat MBP, but no

Figure 15. Ability of SEB peptides to inhibit the proliferation of HPMC stimulated by the superantigen, SEB. Data are expressed in terms of percent reduction ± SE. All peptides were present at a final concentration of 300 μM during the 72 hr incubation with HPMC (5 x 10^5)/well and SEB at a final concentration of 10 pg/ml. The incorporation of radiolabel by cells and SEB was 28,167 ± 8417 and by cells alone was 2953 ± 1584. Values were compared for statistically significant differences using analysis of variance followed by Student's t test.


% Reduction
0
10
20
30
40
50
60
70
80
(1-33)
(31-64)
(60-94)
(90-128)
(124-154)
(150-183)
(179-212)
(208-239)
SEB Peptides
p < 0.02

SEB showed signs of EAE at an mean day of onset of 16.2 (± 2.5) days (Table VI) with a mean severity of 1.8 (± 0.88) (mild paraplegia). By contrast, four out of five mice treated with SEB prior to immunization with rat MBP showed no signs of EAE after 35 days. Only one mouse exhibited signs of EAE at an extended time point of 24 days with a severity of onset of 1 (loss of tail tone). Likewise, five out of six mice in experiment 2 that received rat MBP, but no SEB showed signs of EAE at a mean day of onset of 15.4 (± 2.1) with a mean severity of 1.4 (± 0.49). Three of five mice injected with SEB did not develop signs of EAE while two mice exhibited onset of EAE at days 15 and 19 with a severity of 1 and 5 respectively.

The specific Vß populations of mice that had been protected by SEB treatment were studied next. It has been shown that $Vß8.2^+$ $CD4^+$ T cells are associated with induction of EAE in PL/J mice (Acha-Orbea et al., 1988). Study of spleen cells of SEB treated PL/J mice (no signs of EAE 35 days post immunization) by two-color FACS analysis revealed a reduction of $Vß8^+$ $CD4^+$ T cells (Figure 16). In comparison, analysis of spleen cells from untreated EAE resolved PL/J mice showed the presence of $VB8^+$ $CD4^+$ T cells. A total of four mice/group (SEB protected and EAE resolved) were studied. Inspection of $Vß6^+$ T cells showed no significant difference between SEB treated and untreated EAE resolved mice (data not shown). The Vß profiles of SEB treated and untreated EAE resolved mice correspond to the pattern expected for EAE and the known Vß specificity of SEB.

Presented in Figure 17 is the comparison of the ability of spleen cells from

Table VI. Treatment with SEB prevents development of EAE in PL/J mice*

SEB	Incidence	Mean severity	Mean day of onset
Expt. 1			
+	1/5	1	24
-	5/6	1.8 (±0.88)	16.2 (±2.4)
Expt. 2			
+	2/5	1,5	15,19
-	5/6	1.4 (±0.49)	15.4 (±2.1)

[*] PL/J mice were injected with 300 μg of rat MBP in a 1:1 emulsion of complete Freund's adjuvant, H37Ra (4 mg/ml). Mice received Bordetella pertussis toxin (400 ng) on day of injection and 48 hrs later. SEB treated mice received 40 μg SEB in 0.2 ml PBS i.p. 5 days prior to immunization with rat MBP. Untreated mice received 0.2 ml PBS i.p. 5 days prior to immunization with rat MBP. Mean and standard deviation are given only for groups of mice that contained statistically significant populations. Severity of EAE is represented as a graded scale in which: 0, no signs of EAE; 1, loss of tail tone only; 2, mild paraparesis; 3, severe paraparesis; 4, paraplegia; 5, moribund. Chi-square test of the significance of the difference in incidence between SEB injected and control PL/J mice from the two experiments; $p < 0.012$.

Figure 16. Two-color FACS analysis of PL/J spleen cells showing $V\beta8^+$ $CD4^+$ T cell depletion in SEB protected PL/J mice. Percentage of $V\beta8^+$ $CD4^+$ T cells ± standard error for SEB protected PL/J mice, 2.36 ± 1.32 (A) and EAE resolved PL/J mice, 5.72 ± 0.30 (B). Values given are the mean of three individual experiments. Chi-square test of the significance of reduction between EAE resolved and SEB protected mice; $p < 0.05$. Either SEB treated (A) or untreated EAE resolved (B) were killed and the spleens were removed 40 days after treatment with SEB. Ammonium chloride treated single cell suspensions were analyzed with biotinylated anti-Vβ8 and anti-Vβ6 antibodies and FITC labeled anti-CD4 and -CD8 antibodies. Results for staining with anti-Vβ8 antibodies are presented as individual contour graphs. Staining with anti-Vβ6 antibodies exhibited no significant difference between SEB treated and untreated EAE resolved mice (data not shown).

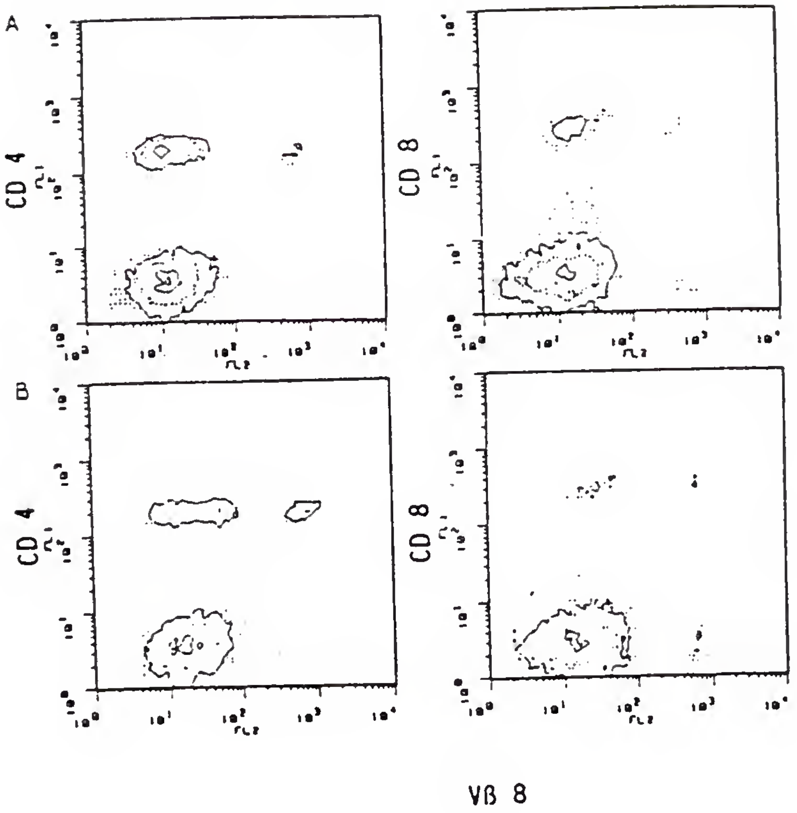
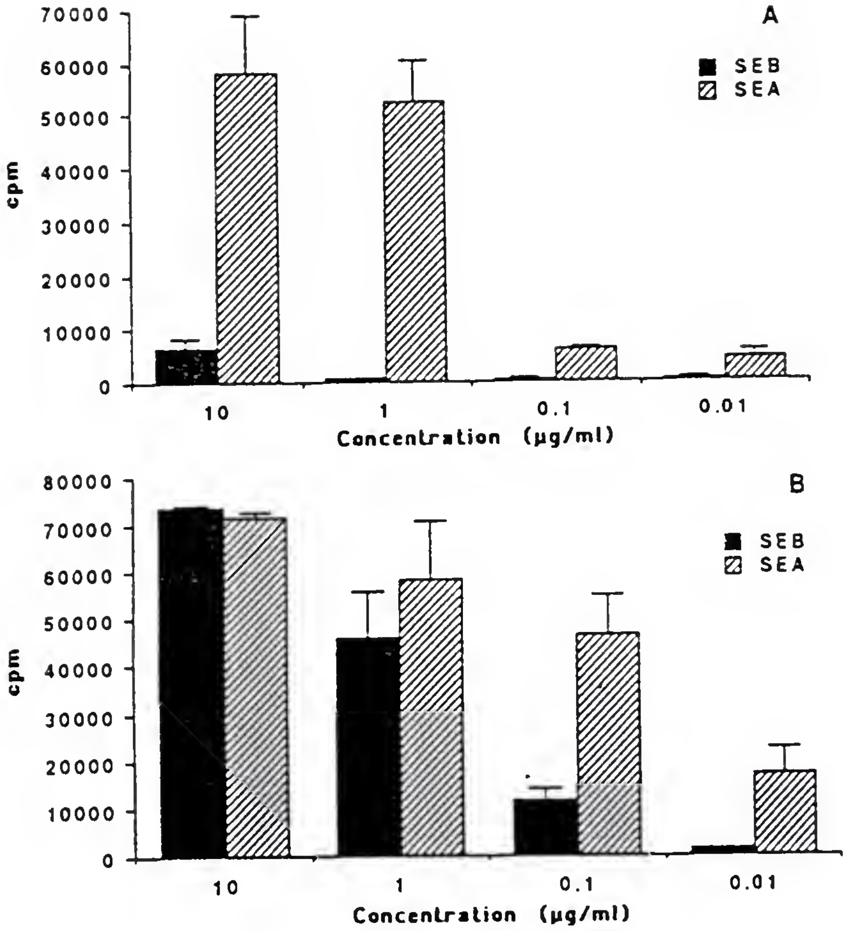

A
B
CD 4
CD 8
CD 4
CD 8
FL1
FL2
10⁰
10¹
10²
10³
10⁴
Vß 8

Figure 17. SEB treated PL/J mice that did not develop EAE are unresponsive to SEB but respond to SEA in vitro (A). Untreated EAE resolved PL/J mice respond to both SEB and SEA in vitro in a dose-dependent manner (B); (closed bars) SEB; (hatched bars) SEA. Proliferation assays were performed 40 days after SEB treatment or PBS treatment alone. Data are indicated as arithmetic means of quadruplicate samples. 5 x 10^4 cells/well were used.


A
■ SEB
▨ SEA
cpm
70000
60000
50000
40000
30000
20000
10000
0
10
1
0.1
0.01
Concentration (µg/ml)
B
■ SEB
▨ SEA
cpm
80000
70000
60000
50000
40000
30000
20000
10000
0
10
1
0.1
0.01
Concentration (µg/ml)

SEB treated mice that did not develop EAE and untreated mice that had resolved all signs of EAE to proliferate in response to SEB and SEA. Spleen cells were stimulated in vitro with 10.0, 1.0, 0.1, 0.01 μg/ml of either SEB or SEA. Cells from mice treated with SEB that did not develop EAE were unresponsive to in vitro stimulation by SEB while stimulation by SEA produced a vigorous response. Such a response to SEA is understandable in that SEA has a Vβ specificity different from SEB, thus it would not be affected by previous exposure of the animal to SEB. In contrast, cells from EAE mice that were not treated with SEB were able to respond to both SEB and SEA in a dose-dependent manner. The greater response to SEA is consistent with the previously reported greater reactivity of this superantigen (Smith and Johnson, 1975). Therefore it would appear that the Vβ specificity of SEB plays an important role in its ability to protect PL/J mice from induction of EAE.

Reactivation of EAE by Staphylococcal Enterotoxins

Six PL/J mice were initially immunized with rat MBP in complete Freunds adjuvant (CFA) followed by pertussis toxin. As can be seen in Figure 18, 5/6 mice developed signs of EAE with a mean severity index of 1.8, which resolved within a week. One month after the development of EAE, when the mice were clinically normal and no other relapse of EAE was noted, four mice were injected with SEB and pertussis toxin only (rat MBP was not repeated). Three weeks following this injection, 2/4 mice developed a second episode of clinical EAE. This episode resolved and the mice were again clinically normal. Two weeks following resolution of all clinical signs the same 4 mice were again injected with SEB and pertussis, with a relapse of the clinical signs of EAE seen in 3/4 mice (onset 1-2 weeks post injection) (Figure 18 and Table VII, Experiment 1). A similar result was seen in an additional experiment (Table VII, Experiment 2). No relapses occurred when mice were injected with pertussis only. Another group of 15 PL/J mice were administered SEB (40 μg i.p.) (mice had not been previously immunized with rat MBP), and none of them developed evidence of encephalitis (data not shown).

Next, we wished to determine whether SEB could re-induce EAE in the absence of pertussis toxin. In a total of four mice that had previously been immunized with MBP and received SEB plus pertussis toxin, two developed clinical signs of EAE (Table VIII, Experiment 1) with SEB only. However, the severity and duration of clinical signs was less than previous episodes in mice re-induced with SEB only (compared to SEB and pertussis). Nevertheless, SEB alone was capable of re-inducing EAE in some mice that had previously received SEB and pertussis toxin.

Finally, we evaluated whether EAE could be induced in those mice that were immunized with rat MBP in CFA and pertussis toxin, but who never developed clinical evidence of EAE. A total of 3/7 mice developed EAE after injection with SEB and pertussis toxin (Table VII, Experiment 3), and of those 3, one animal developed EAE after re-injection with SEB only. Thus, SEB is able to induce EAE in mice immunized with rat MBP but who did not develop clinical signs of EAE.

The finding that mice injected repeatedly with SEB developed clinical EAE following each injection was unexpected. Although SEB can activate $V\beta8^+$ cells, it has been demonstrated that following this period of activation the $V\beta8^+$ T cells become unresponsive to further stimulation with SEB (or to stimulation with anti $V\beta8^+$ antibodies) (Kawabe and Ochi, 1990; Rellahan et al., 1990; Kawabe and Ochi, 1991). We reasoned that either $V\beta8^+$ T cells that were previously activated must be resistant to the development of anergy upon stimulation with SEB, or that the pertussis toxin injected along with the SEB prevented the induction of anergy by SEB. Figure 19, Panels A and B demonstrate that T cells activated in vivo with rat MBP in CFA are indeed resistant to the induction of anergy. In Panel A, mice were administered either SEB one week before immunization with rat MBP in CFA or administered SEB one week after immunization with rat MBP in CFA. $V\beta8^+$ proliferation was evaluated by stimulation of T cells for three days in microtiter wells coated with an anti-$V\beta8$ antibody followed by ^{3}H-thymidine incorporation. $V\beta8^+$ T cells obtained from animals administered SEB one week before immunization with rat MBP in CFA exhibited a reduced response, while $V\beta8^+$ T cells from mice administered SEB one week after immunization with rat MBP in CFA were not anergized. Interestingly, mice that were immunized with only CFA and received SEB one week later were also not anergized (data not shown). Controls consisted of PL/J mice immunized with only rat MBP in CFA. T cells from all three groups proliferated equally well when stimulated with an anti-$V\beta9^+$ antibody in vitro (data not shown), suggesting that $V\beta8$ T cells had been specifically anergized. In addition, in Panel B we evaluated $V\beta8^+$ T cell proliferation in one group of mice that had previously developed a clinical episode of EAE and who were administered SEB one month after the episode of EAE. $V\beta 8^+$ T cells from these mice also failed to be anergized after in vivo exposure to SEB. These results show that the timing of administration of SEB determines whether cells are anergized or activated. Thus, it appears that $V\beta 8^+$ T cells stimulated in vivo by rat MBP and CFA or CFA alone are resistant to the induction of anergy by SEB.

Figure 19 demonstrates that pertussis toxin cannot overcome the anergizing effects of SEB when injected simultaneously with SEB in previously unimmunized animals although pertussis toxin itself is not a superantigen (Kamradt et al., 1991). It had previously been demonstrated that pertussis toxin could prevent the induction of T cell anergy to an encephalitogenic peptide of MBP injected I.V. and that pertussis toxin has strong mitogenic effects (Kamradt et al., 1991). However, pertussis toxin, when injected simultaneously with SEB, was not able to overcome SEB induced T cell anergy.

While SEB can activate $V\beta 8^+$ T cells, SEA cannot. In order to determine whether reactivation of EAE was specific for SEB, we immunized a separate group of PL/J mice with rat MBP and pertussis toxin. Six out of 10 mice developed EAE. Following remission of clinical EAE, mice were then injected with SEA and pertussis toxin. The 6 EAE mice developed a clinical relapse of EAE (Table VIII). The 4 mice that were immunized with MBP but that did not develop clinical symptoms were also injected with SEA (40μg i.p.) and pertussis toxin one month after resolution of clinical symptoms. A total of 2/4 of these mice exhibited clinical

Figure 18. Time course of reinduction of EAE in PL/J mice initially immunized with rat MBP in CFA and pertussis and given multiple injections of SEB. A relapse of EAE occurred each time following immunization with either SEB and pertussis or SEB alone.

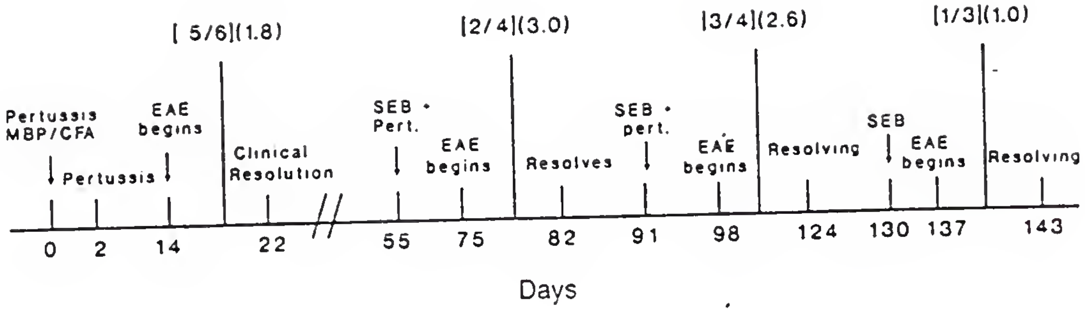

[5/6](1.8)
[2/4](3.0)
[3/4](2.6)
[1/3](1.0)
Pertussis MBP/CFA
Pertussis
EAE begins
Clinical Resolution
SEB + Pert.
EAE begins
Resolves
SEB + pert.
EAE begins
Resolving
SEB
EAE begins
Resolving
0
2
14
22
55
75
82
91
98
124
130
137
143
Days

Table VII. Reactivation of EAE with SEB

	Incidence of disease, no. mice with EAE/total no. injected			
Exp.	Immunization with rat MBP	First injection SEB plus pertussis	Second injection SEB plus pertussis	Third injection SEB only
1	5/6(1.8)	2/4(3.0)*	3/4(2.6)*	1/3(1.0)
2	3/3(2.0)	1/3(3.0)	-	1/1(2.0)
3	0/7**	3/7(3.7)	-	1/3(2.0)

All mice in all experiments were initial immunized with MBP in CFA and pertussis to induce EAE. Some mice, however, never developed clinical signs of EAE. Whether or not mice developed EAE, they were subsequently administered SEB (40 μg i.p.) and evaluated for clinical signs of EAE. Numbers in parentheses are mean severity index.

* One mouse that did not develop EAE after the first injection of SEB and pertussis developed EAE after the second injection.

** These mice were pooled from several previous experiments in which mice were immunized with MBP and pertussis. Although some mice developed clinical EAE, the seven mice used in this experiment never developed clinical signs of EAE.

Figure 19. Induction of T cell anergy by superantigen is prevented by previous activation. (A) Bars: 1, group was immunized with MBP only; 2, group received SEB (40 μg i.p.) and was immunized 1 week later with MBP; 3, group was immunized with MBP and received SEB (40μg i.p.) 1 week later. All groups were sacrificed 1 week after the last immunization or injection. Procedures were timed for sacrifice of all groups on the same day. Proliferation was induced with anti Vß8 antibodies and measured by [^{3}H]thymidine incorporation (Rellahan et al., 1990). Group means differed significantly by an ANOVA ($P>0.0031$). (B) Bars: 1, group received SEB (40 μg i.p.) only; 2, group received both toxins (SEB at 40 μg and pertussis toxin at 500 ng i.p.) simultaneously; 3, group was immunized with MBP and i week later received SEB (40 μg i.p.); 4, group was immunized for induction of EAE and developed acute symptoms, and symptoms were resolved. One week after resolution mice received SEB (i.p.). All groups were sacrificed 1 week after the last immunization or injection. Again, procedures were timed for sacrifice of all groups on the same day. Two to three mice were used per group per experiment and proliferation was induced with anti-Vß8 antibodies. Significance between groups 1 and 2 versus groups 3 and 4 was determined by an ANOVA ($P<0.0001$). Controls were performed for all experiments by measuring proliferation induced by anti-Vß9 antibodies or in the absence of antibody. Cells from all groups proliferated equally well when stimulated with anti-Vß9 antibodies.

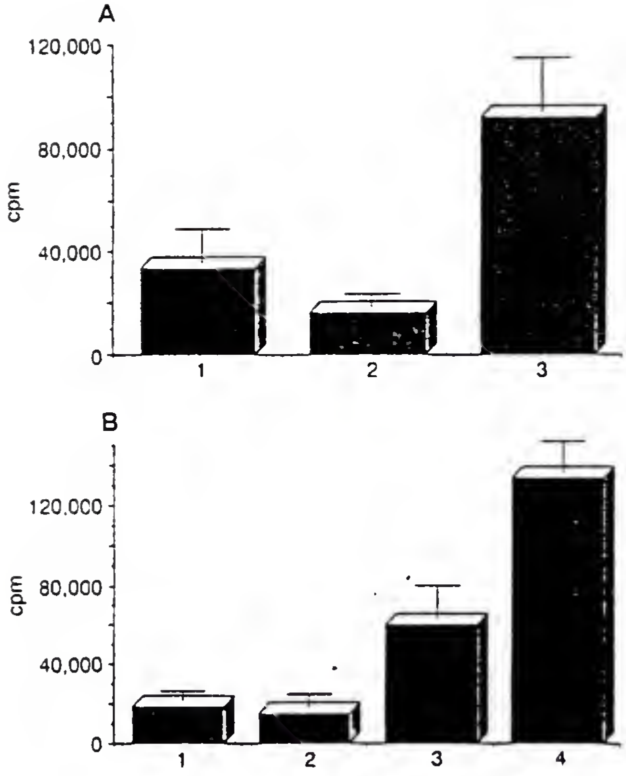

A
120,000
80,000
40,000
0
cpm
1
2
3
B
120,000
80,000
40,000
0
cpm
1
2
3
4

Table VIII. Reactivation of EAE by SEA

Incidence of disease, no. of mice with EAE/total no. injected

Initial development of EAE	Immunization with rat MBP	Injection SEA plus pertussis
Yes	6/6 (2.3)	6/6 (2.9)
No	0/4	2/4 (3.0)

PL/J mice were injected with SEA (40μg i.p.) 1 month after resolution of clinical signs of EAE. Numbers in parentheses are mean severity index.

evidence of EAE (Table VIII). Thus, like SEB, SEA can induce EAE in mice that developed clinical signs of EAE and those mice that were immunized with MBP but did not develop clinical symptoms.

Type I IFN Inhibition of Superantigen Activity

The type I IFNs, HuIFNß, HuIFNα and BoIFNτ, inhibited superantigen-induced proliferation of HPMC. At a final dose of 1000 units/ml, the type I IFNs inhibited T cell proliferation by SEB, SEA and TSST-1 to the same extent, approximately 50 % (Figure 20). The inhibitive effects of BoIFNτ upon superantigen activation was comparable to that of both HuIFNα and HuIFNß, suggesting that BoIFNτ is able to suppress superantigen-driven T cell proliferation across species. In comparison, type II HuIFNγ exhibited no anti-proliferative effects upon superantigen-induced activation. The inhibition of SEB-induced T cell proliferation by the type I IFNs occurred in a dose dependent manner (Figure 21). Thus, the type I IFNs exert an antiproliferative effect on T lymphocytes activated by superantigens.

Dose response studies of SEB-induced activation in the presence of the various type I IFNs indicated that the type I IFNs were effective even at high concentrations of SEB (Figure 22). At a dose of 1000 units/ml, HuIFNs α and ß as well as BoIFNτ consistently inhibited proliferation induced by a wide range of SEB concentrations (10 ng/ml to 5 μg/ml). Therefore, increased concentrations of superantigen were unable to overcome the inhibitive effects exerted by the type I IFNs. In addition, IL-2 production, a characteristic hallmark of T cell activation by superantigen, was also reduced in the presence of the type I IFNs when HPMC were stimulated with SEB (Figure 23). IL-2 resulting from activation of HPMC by superantigen was reduced by approximately 50 % by the type I IFNs with BoIFNτ exhibiting potent cross species activity.

We next determined whether the type I IFNs were capable of greater inhibition of superantigen induced activation when examined in combination with each other (Figure 24). Of the three tested, all of the type I IFNs were as effective individually as when tested in combination. Levels of inhibition of SEB-induced T cell proliferation were approximately 50 % for all of the IFNs except HuIFNγ. That the type I IFNs do not synergize in their effects suggests that their ability to inhibit proliferation occurs via a similar mechanism.

It has been shown previously that superantigens stimulate T cells by binding to the Vβ region of the T cell receptor, resulting in an increased population of Vβ specific T cell subsets (White et al., 1989). The Vβ profiles of cultures stimulated with SEB in the presence or absence of the type I IFNs were examined using flow cytometry. Such experiments were conducted to determine if reduction of a specific Vβ T cell subset occurred in accordance with inhibition of SEB-induced activation by the type I IFNs. It was observed that cultures treated with both SEB and type I IFNs exhibited percentages of Vβ12 T cells marginally increased as compared to cells cultured in media only, while cultures treated with SEB alone exhibited a greater than 2-fold increase in the percentage of Vβ12 T cells (Table IX). Another Vβ subset known not to be activated by SEB, Vβ22, exhibited no differences in cell percentages between treatment with SEB in the presence or absence of the type I IFNs. Thus, inhibition of superantigen-induced activation by the type I IFNs occurred in a Vβ specific manner.

We next addressed the question of toxicity induced by high doses of the type I IFNs. Human lymphocytes were treated at a very high dose (50,000 units/ml) of HuIFNα and β and BoIFNτ for 48 hr and examined for cell viability as assessed by trypan blue dye exclusion. As presented in Table X, lymphocytes treated with HuIFNα and β exhibited significantly reduced viability that was not seen in the BoIFNτ treated cells. This observation is consistent with a report of a lack of toxicity by BoIFNτ at high doses using human WISH and bovine MDBK cell lines (Pontzer et al., 1991a). Therefore, it appears that BoIFNτ is an effective antiproliferative agent which may be used at high doses that would normally be toxic for other type I IFNs.

Figure 20. Type I IFNs inhibit superantigen-induced activation of HPMC. Type I IFNs, HuIFNα, HuIFNβ and BoIFNτ, and type II HuIFNγ were added at a final concentration of 1000 units/ml to 2.5 x 10^5 HPMC/well concomitantly with 1 ng/ml SEB (closed bars), SEA (open bars) or TSST-1 (hatched bars). Values are expressed as percent control ± SE at which 100 % represents maximal stimulation by superantigen in the absence of IFN. The results of one of four replicate experiments are presented.

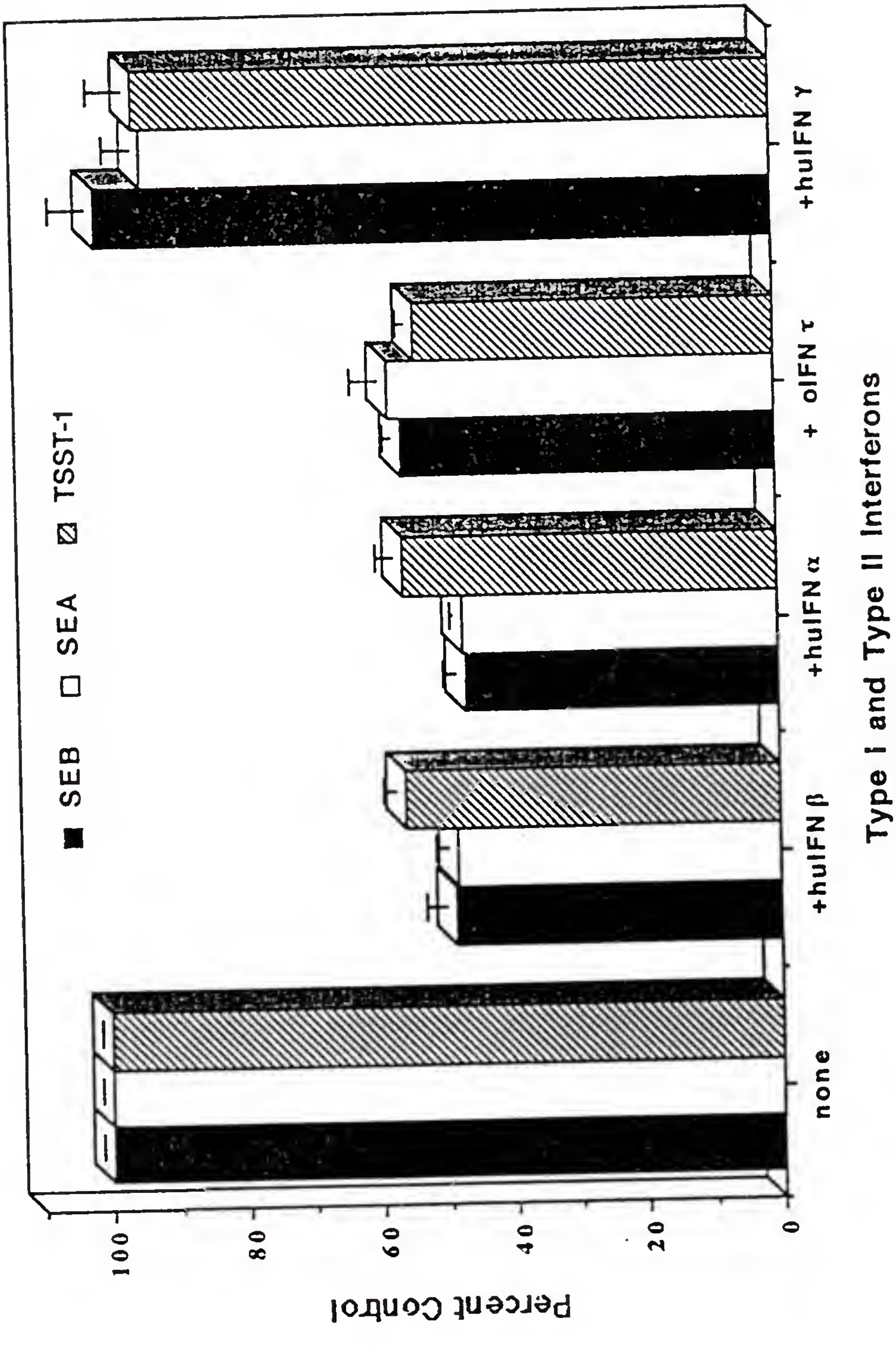

■ SEB
□ SEA
▨ TSST-1
Percent Control
0
20
40
60
80
100
none
+huIFN β
+huIFN α
+ oIFN τ
+huIFN γ
Type I and Type II Interferons

Figure 21. Dose dependent inhibition of superantigen-induced activation by type I IFNs. HPMC were cultured with SEB and multiple doses (10, 100, 1000 and 10,000 units/ml) of HuIFNβ (closed triangle), HuIFNα (closed square), BoIFNτ (closed circle) and HuIFNγ (open circle). Values are expressed as percent control ± SE. The results of one of three replicate experiments are presented.

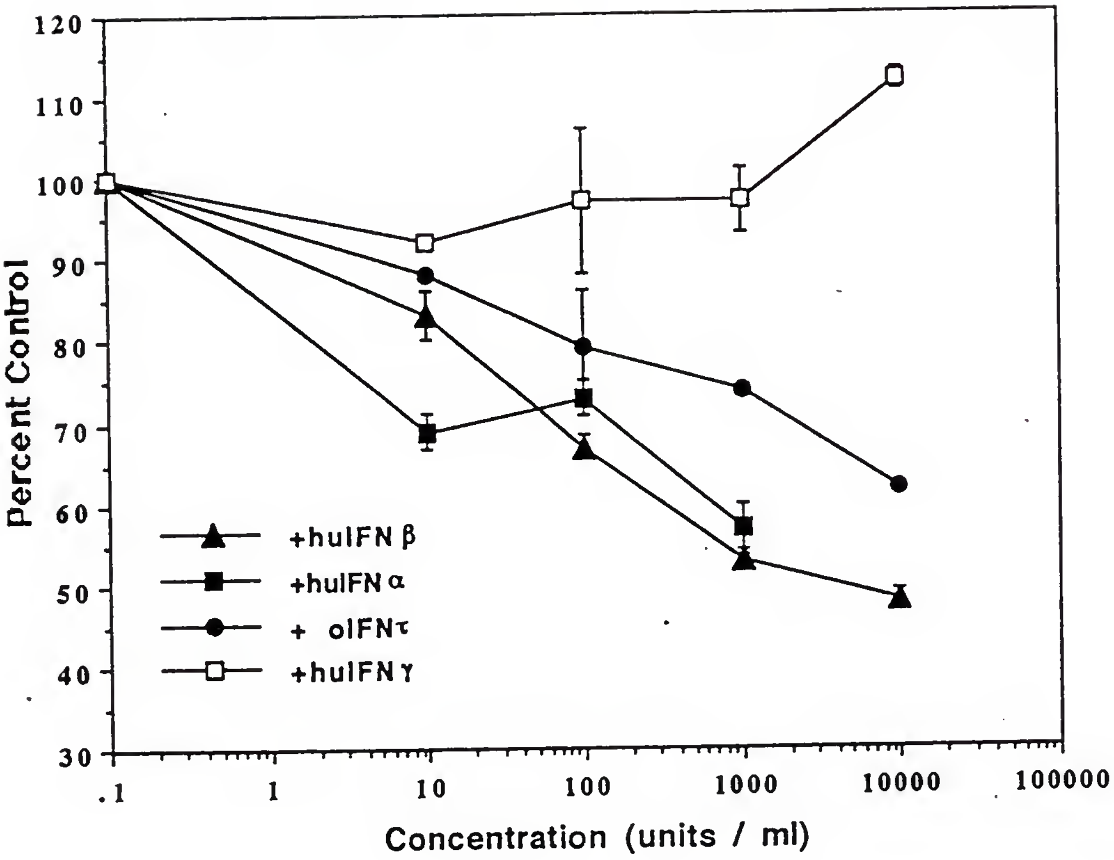

120
110
100
90
80
70
60
50
40
30
Percent Control
.1
1
10
100
1000
10000
100000
Concentration (units / ml)
+huIFN β
+huIFN α
+ oIFN τ
+huIFN γ

Figure 22. Type I IFNs inhibit proliferation driven by high concentrations of SEB. HPMC were cultured with type I IFNs (1000 units/ml) and varying concentrations of SEB ranging from 10 ng/ml to 5 μg/ml. Values are expressed as mean cpm ± SEM and shown as SEB alone (closed square), +HuIFNβ (closed triangle), +HuIFNα (closed circle) and +BoIFNτ (open circles).

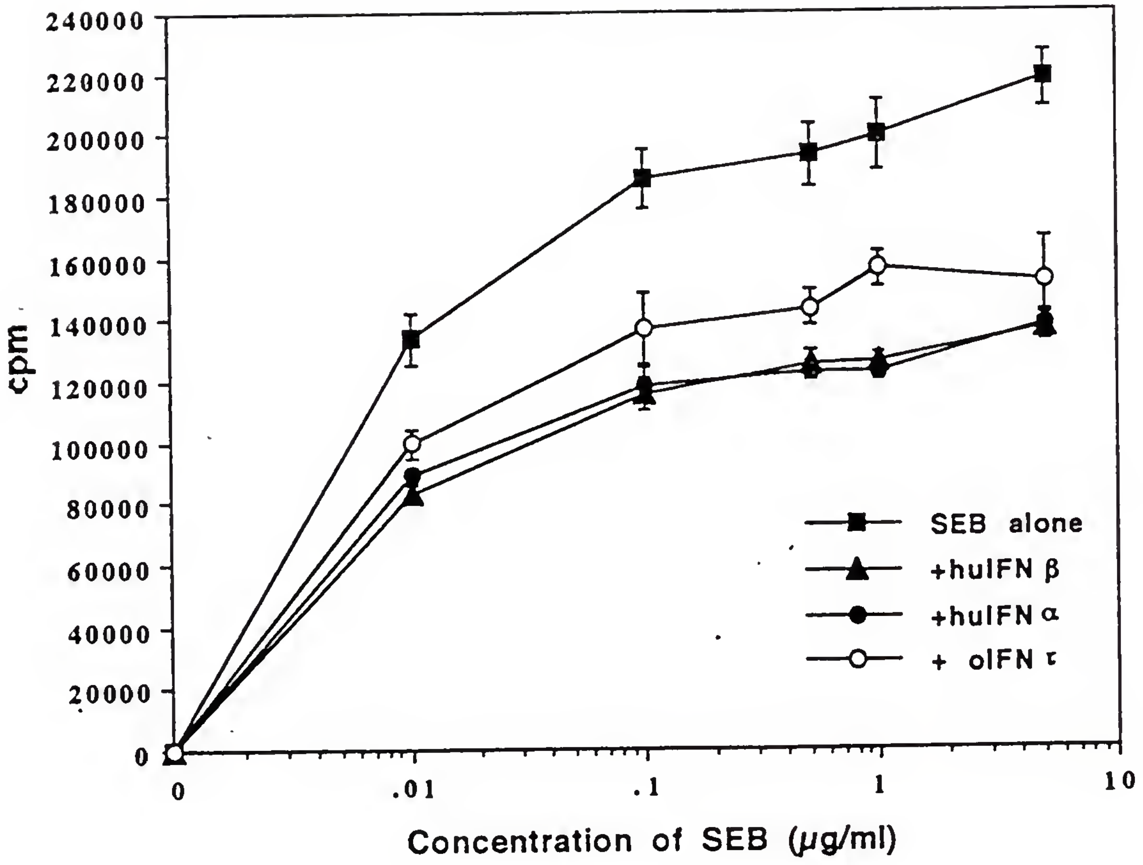

240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
cpm
0
.01
.1
1
10
Concentration of SEB (μg/ml)
SEB alone
+huIFN β
+huIFN α
+ oIFN τ

Figure 23. Inhibition of IL-2 activity of HPMC cultured with SEB in the presence or absence of type I IFNs. Supernatants after 48 hr stimulation with SEB and IFNs were examined for IL-2 activity using the IL-2 dependent cell line HT-2. Values are expressed as mean ± SEM.

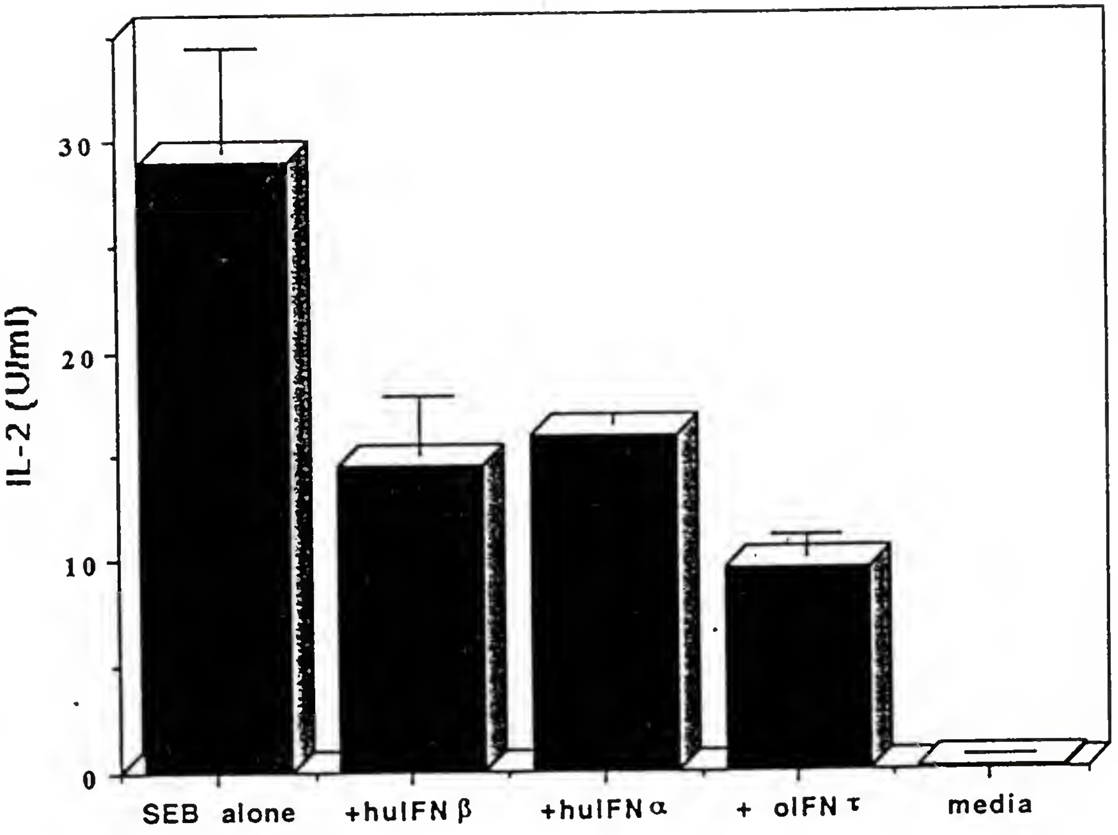

IL-2 (U/ml)
30
20
10
0
SEB alone
+huIFN β
+huIFN α
+ oIFN τ
media

Figure 24. Type I IFNs individually inhibit SEB-induced proliferation to a similar degree as when tested in combination. IFNs tested were added at a final concentration of 1000 units/ml to HPMC cultured with SEB (1 ng/ml). Values are expressed as percent control ± SE.

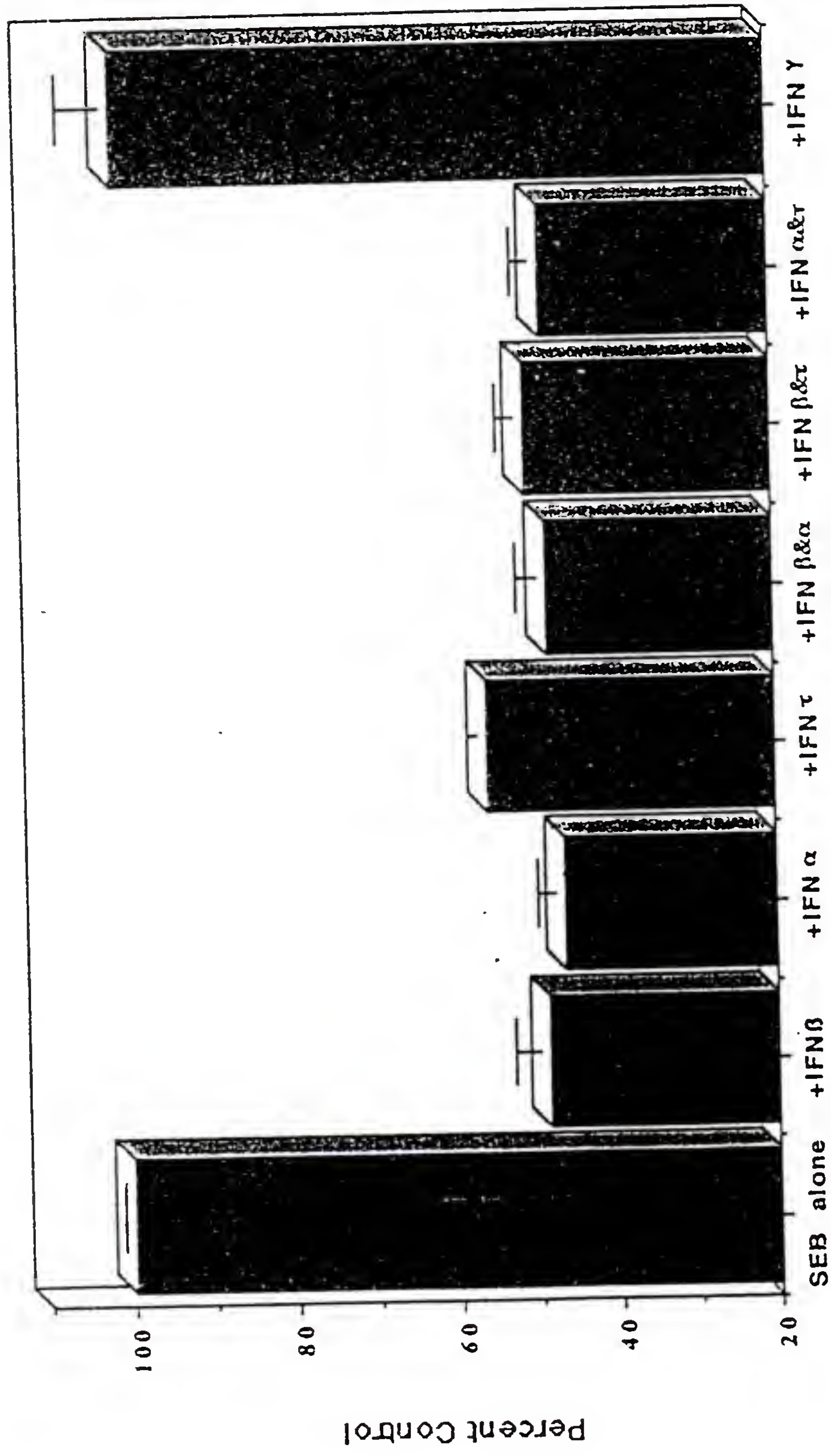

Percent Control
20
40
60
80
100
SEB alone
+IFNß
+IFN α
+IFN τ
+IFN β&α
+IFN β&τ
+IFN α&τ
+IFN γ

Table IX. Inhibition of Vβ specific SEB-induced T cell proliferation by the type I IFNs as assessed by flow cytometry.[*]

Vβ	Treatment				
	media	SEB	+HuIFN-β	+HuIFN-α	+BoIFN-τ
Vβ 12	7.4 ± 0.1	15.9 ± 1.8	8.8 ± 0.9[**]	10.3 ± 0.4[**]	10.4 ± 1.7[**]
Vβ 22	5.2 ± 0.7	5.8 ± 0.4	4.8 ± 0.3	4.4 ± 0.5	4.0 ± 2.0

[*] Cultures were analyzed after 72 hr treatment with SEB alone, SEB in the presence of the type I IFNs (1000 U/ml), or media alone. Values are expressed as percentage of $V\beta^+$ T cells ± standard error.

[**] Significance of the reduction between SEB treated HPMC and HPMC treated with SEB and type I IFNs as measured by student's t test : $p < 0.03$.

Table X. IFNτ is less cytotoxic than other IFNs.*

Treatment	Cell no. ($\times 10^5$)		
	Live	Dead	Viability (%)
Media	6.4 ± 0.3	0.7 ± 0.1	91
BoIFNτ	5.3 ± 0.5	1.0 ± 0.2	85
HuIFNβ	4.3 ± 0.5	2.0 ± 0.2**	69
HuIFNα	4.3 ± 0.6	2.3 ± 0.1**	66

* HPMC were incubated with IFNs (50,000 units/ml final concentration) and at 48 hr triplicate wells were harvested for viability determinations.

** $P < 0.05$ as determined by student's t test.

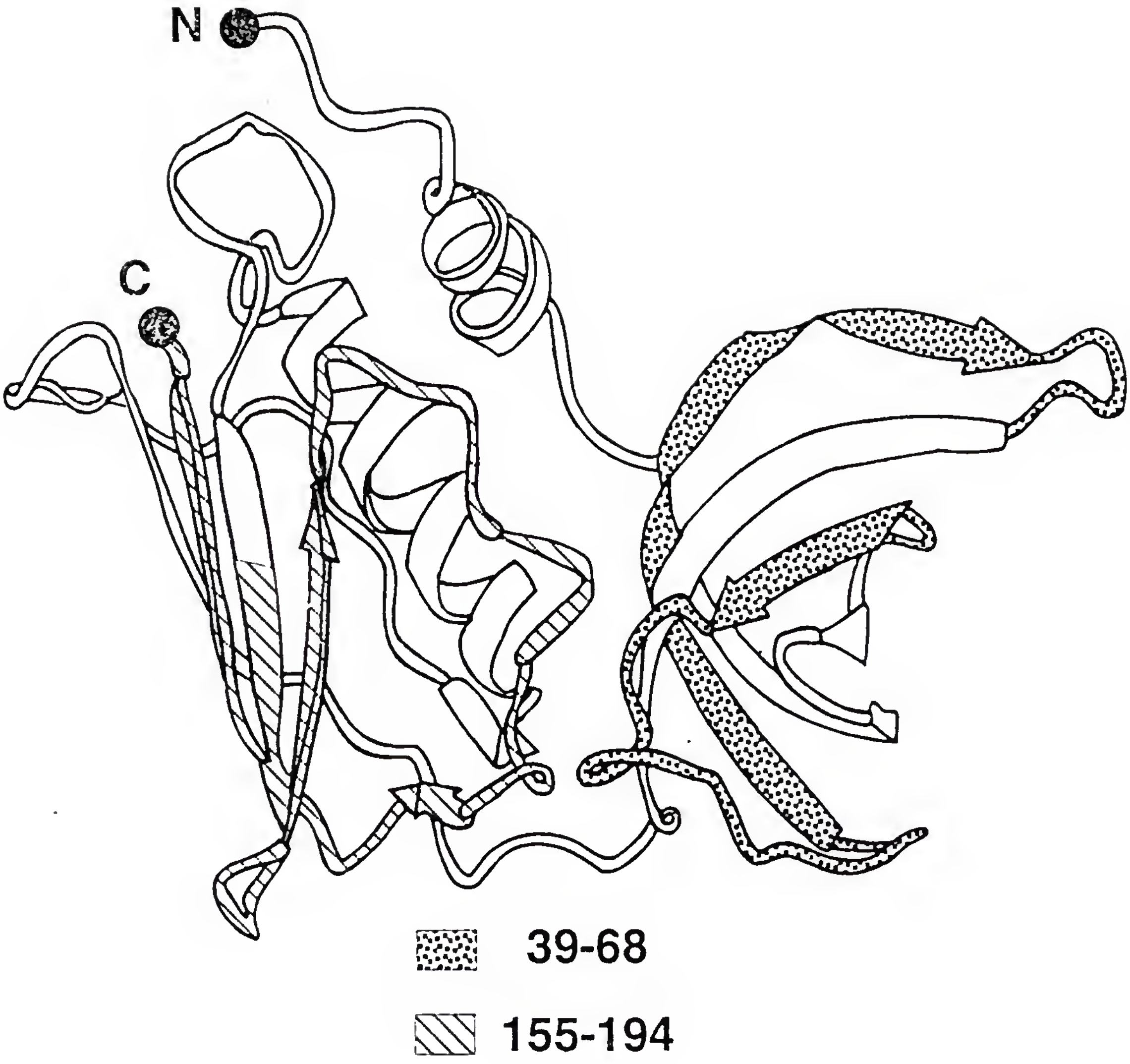
DISCUSSION

Structural Studies

The synthetic peptide approach was employed for the identification of MHC class II molecule binding sites on two superantigens, TSST-1 and SEB. In the case of TSST-1, the synthesis of peptides was initiated prior to the determination of the TSST-1 crystal structure. Peptides were designed based on the hydropathic plot and primary sequence of TSST-1. By peptide competition studies, regions of TSST-1 encompassing amino acids 39 through 68 and 155 through 194 were shown to be involved in binding to the class II MHC molecule. We theorized that these two domains may potentially serve as individual sites of TSST-1 binding to its receptor or as a single non-contiguous MHC class II binding domain of TSST-1. Epitopes for monoclonal antibodies with neutralizing activity have been mapped to a large 14,000 dalton internal fragment of TSST-1 (Blomster-Hautamaa et al., 1986b). A synthetic decapeptide encompassing the region 34 through 43 of TSST-1 blocked the neutralizing activity of a monoclonal antibody to TSST-1 in TSST-1 induced T-cell mitogenesis (Murphy et al., 1988). It has also been shown that the N-terminal region of TSST-1, encompassing amino acids 34 through 63 can serve as specific antigen, but a peptide corresponding to this region did not compete with TSST-1 for binding to class II MHC molecules (Ramesh et al., 1992). In addition, chemical modification of histidine and tyrosine residues of TSST-1 has also been shown to inhibit the toxin's mitogenic activity (Kokan-Moore et al., 1989). Therefore, the data presented here is the first direct evidence for domains of TSST-1 involved in binding to class II MHC molecules.

The TSST-1 molecule contains relatively high ß-structure and aperiodic structure with relatively low α-helical content as determined by circular dichroism (Singh et al., 1988b). Of the overlapping TSST-1 peptides, ß-structure was found to be the predominant structure over α-helix among all the peptides. Determination of the TSST-1 crystal structure has allowed for the placement of MHC class II binding regions within the molecule's structure (Figure 25). Residues 39-68 of the N-terminus of TSST-1 important for MHC class II binding are present in the secondary structures ß3 and ß4 while residues of the C-terminus important for binding MHC class II reside in the secondary structures ß9, ß10, ß11 and ß12. The superantigen activity of TSST-1 appears to cluster into two main sites. Site A, composed of the concave face of the N-terminal ß-barrel and residues between 170 and 180 of the C-terminus which pack closely against the ß-barrel, is associated with binding to MHC class II. Although we have identified two TSST-1 peptides able to compete with TSST-1 for binding to MHC class II, these peptides may possibly form a noncontiguous binding site within the structure of TSST-1. It has been suggested that a weakness of the synthetic peptide approach is its inability to identify noncontiguous binding sites. However, we have successfully determined regions of TSST-1 involved in MHC class II interaction even though these regions ultimately formed a noncontiguous site. Whether these regions of

Figure 25. Location of MHC class II binding regions within a ribbon model of the crystal structure of TSST-1. Residues 39-78 are colored yellow and residues 155-194 are colored green. These regions form the noncontiguous, site A, responsible for TSST-1 interaction with MHC class II.


N
C
░ 39-68
▧ 155-194

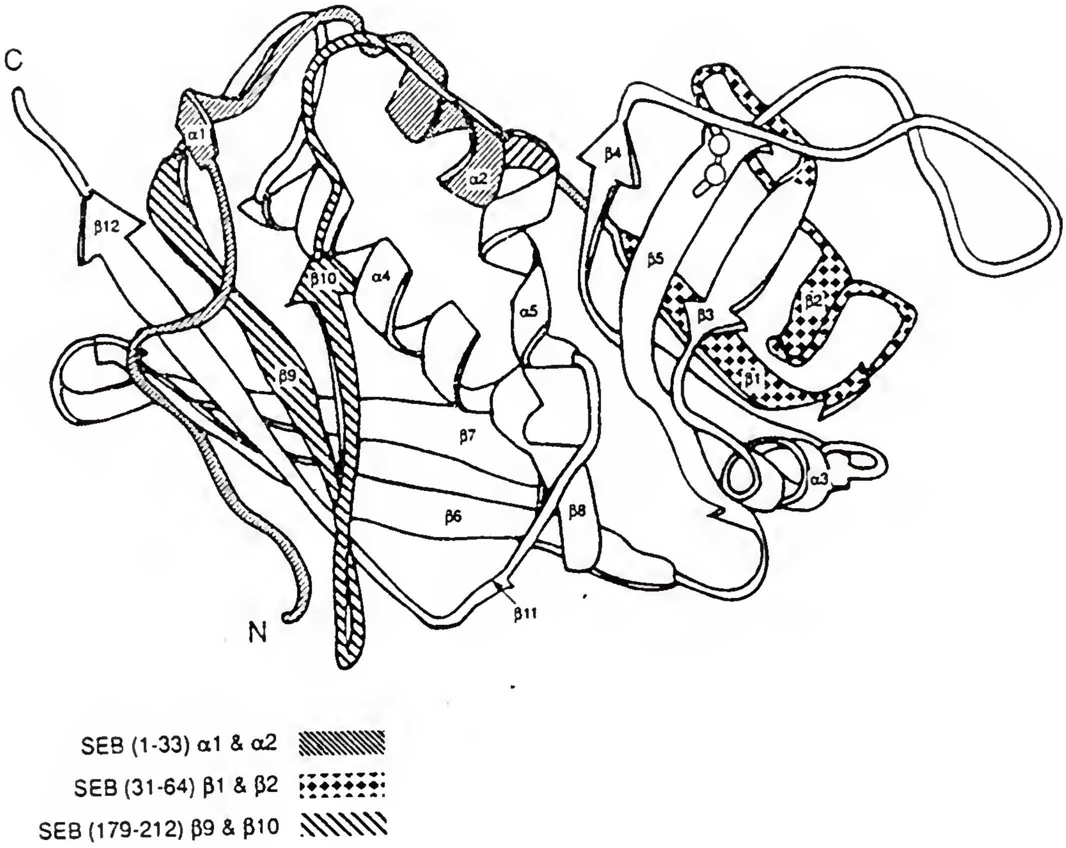
the TSST-1 molecule remain a noncontiguous site in the bound molecule has yet to be determined. The second region,site B, composed of α2, β7, β8 and α2β9 loops of the C-terminal domain appears to be the likely site of TCR binding although direct evidence for this interaction is as yet unavailable.

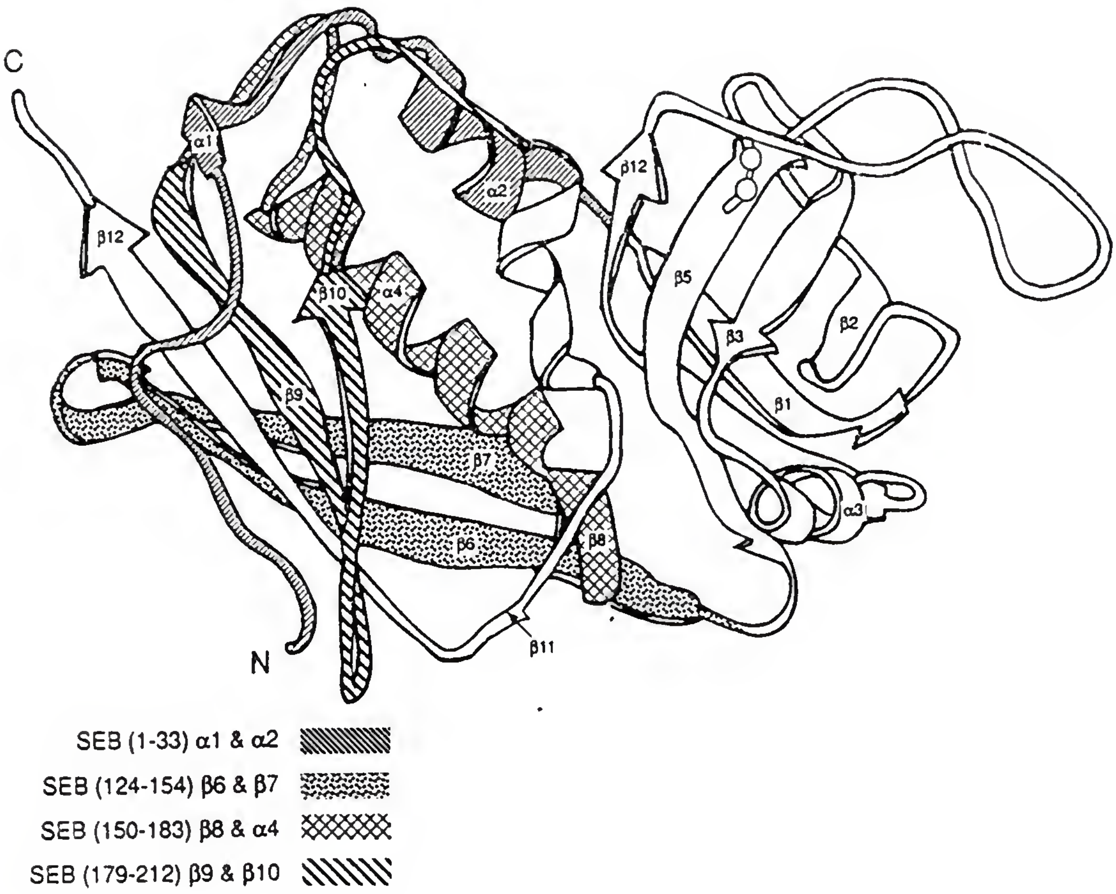
We have also shown that the class II MHC peptides I-A_{β}^{b}(30-60) and I-A_{β}^{b}(60-90), which encompass a β-turn and the entire β-chain α-helix respectively (Bjorkman et al., 1987; Brown et al., 1988), inhibit binding of TSST-1 to both Raji and A20 cells by peptide competition studies. A previous study showed the importance of the α-chain for binding to TSST-1 (Karp et al., 1990). Thus, TSST-1 appears to bind to combined residues of the α-chain and β-chain of the class II MHC molecule.

In the case of SEB, the approach taken in designing the overlapping synthetic peptides was different from that used for the TSST-1 studies. With the advantage of having the SEB crystal structure in hand, the SEB peptides were designed to encompass discrete secondary structures present in the 3-dimensional structure of SEB. The predicted composite surface profile of SEB was also taken into consideration. We have demonstrated here that the manner in which SEB interacts with its receptor, the MHC class II molecule, may be dependent upon the haplotype of the MHC class II molecule involved. It was shown that different as well as overlapping regions of the SEB molecule are responsible for its interactions with receptor on DR1 transfected L cells and Raji cells. In addition, we have further localized regions present in the carboxy-terminus of the SEB molecule involved in binding to MHC class II molecules. The regions 1-33, 31-64, and 179-212 of SEB are involved in binding to DR1 transfected L cells. In comparison, the regions 1-33, 124-154, 150-183, and 179-212 are involved in binding to DR3, DRw10, DQw1 and DQw2 on Raji cells. Interestingly, we found that only 20 to 40 μM of peptide were required to inhibit 50 % of SEB binding to Raji cells as compared to 100 μM of peptide required for DR1 transfected L cells. This difference may be due to the reported greater amount of binding by SEB to DR3 and DRw10 as compared to DR1 (Scholl et al., 1990). In addition, a truncation of SEB (179-212) encompassing residues 186 through 212 was also able to compete with SEB for binding to DR 1 transfected L cells and Raji cells, while further truncations of SEB (179-212) exhibited a gradual lack of competition. Thus, the finding that different regions of SEB are important for binding to different haplotypes of HLA class II molecules suggests that the binding of the staphylococcal enterotoxins is more complex than previously thought.

Recently, the three dimensional crystalline structure of SEB was determined and was shown to consist of a two domain general motif for the staphylococcal enterotoxins (Swaminathan et al., 1992). The regions of SEB involved in binding to DR1 transfected L cells appear to be present in both domains of the structure of SEB as shown in the shaded regions of the ribbon structure of Figure 26A. By comparison, regions of SEB involved in binding to the MHC class II molecules present on Raji cells appear to be contained within a single domain of the molecule (Figure 26B). The MHC class II binding sites in SEB and probably other

Figure 26. Locations of regions of SEB important for binding to MHC class II molecules. A ribbon model of the three dimensional crystalline structure of the SEB molecule is shown with regions important for binding to DR 1 transfected L cells (A) and Raji (B) shaded.


C
α1
α2
β4
β12
β10
α4
α5
β5
β3
β2
β9
β1
β7
α3
β6
β8
N
β11
SEB (1-33) α1 & α2 ▨
SEB (31-64) β1 & β2 ▦
SEB (179-212) β9 & β10 ▧


C
α1
α2
β12
β12
β10
α4
β5
β3
β2
β1
β9
β7
β6
β8
α3
β11
N
SEB (1-33) α1 & α2 ▨
SEB (124-154) β6 & β7 ▒
SEB (150-183) β8 & α4 ▩
SEB (179-212) β9 & β10 ▧

staphylococcal enterotoxins appear to be present at multiple sites in the SEB molecule, and are not localized to a single site.

Our laboratory has previously identified regions important for MHC class II molecule binding on SEA (Pontzer et al., 1990; Griggs et al., 1992). Assuming that the structures of SEB and TSST-1 are a generalized two domain structure that, in the case of SEB, applies to the other staphylococcal enterotoxins, it is certainly of interest to compare what is known about other superantigens with the known superantigen crystal structures. It has been shown that the residues of SEA that are important for MHC class II binding are 1-86 and 121-149. The region 1-86 of SEA would be present in both domains of the molecule, while 121-149 would be localized to a single domain. That multiple binding sites for MHC class II are present on superantigens such as SEA and SEB in either a single domain or both domains of the molecule may help to explain the lack of species or haplotype specificity of superantigen binding and their versatility in the activation of T cells.

In order to localize regions of SEB important for mitogenic function, the ability of the synthetic SEB peptides to inhibit SEB-induced proliferation of HPMC was also examined. A previous study showed that an SEB fusion protein containing residues 1-130 did not exhibit mitogenic activity while a different fusion protein containing residues 1-138 did stimulate T cells (Buelow et al., 1992). The residues 131-138 are contained in the SEB peptide (124-154), supporting the importance of this region of SEB for mitogenic activity. Whether this region is important for MHC class II molecule binding only, or that it may also contain residues important for contact with the TCR remains to be determined.

Although staphylococcal enterotoxin superantigens do not exhibit MHC restriction, they do show haplotype preference. It has been shown, for example, that HLA-DR haplotypes vary in their ability to bind and present some of the staphylococcal enterotoxins (Herman et al., 1990; Scholl et al., 1990). It has also been shown that the haplotype of the MHC class II molecule responsible for presentation of a superantigen can affect the degree of stimulation of specific Vβ bearing T cells (B. Cole, personal communication). We suggest that different regions of SEB may also be involved in binding to various alleles of HLA-DR as well as the DQ molecules present on Raji cells. The binding of different regions of SEB to MHC class II molecules would suggest that the Vβ regions of the T cell receptor may not all recognize a given superantigen complexed to different MHC class II molecules in the same way. Thus, the superantigen Vβ specificity may vary depending on the MHC class II molecule involved in presentation. It would thus appear that the contacts in the MHC/SAg/TCR complex are quite dynamic, depending upon the nature of the binding of superantigen to MHC class II molecules.

Role of Superantigens in Experimental Allergic Encephalomyelitis

Recently, it was shown that the response of the murine strain PL/J to MBP and its N-terminal peptide is oligoclonal in nature, specifically T cells bearing Vβ8 TCR. Based on this observation and the known Vβ specificities of the staphylococcal enterotoxins, we have examined the role of bacterial superantigen in EAE. We first studied the effect of treatment with SEB prior to immunization with MBP for EAE induction. We have shown that PL/J mice can be protected from the development of EAE by treatment with SEB. Inspection of the Vß profiles of SEB treated mice that did not develop EAE revealed a 59 % reduction of $Vß8^+$ $CD4^+$ T cells, similar to that reported by others (Kawabe and Ochi, 1991). Spleen cells of these mice were also unresponsive to SEB in vitro but produced a vigorous response to SEA. In comparison, $Vß8^+$ $CD4^+$ T cells were present and responsive to both SEB and SEA in vitro in untreated PL/J mice that had resolved signs of EAE. Therefore, anergy and/or depletion of $Vß8^+$ $CD4^+$ T cells by SEB may be greatly involved in the protection against EAE. Future studies will determine the responsiveness to SEB of the T cells of the few mice that did develop EAE after treatment with SEB.

Recently, several novel immunological approaches have been developed to prevent or treat autoimmune diseases such as EAE in mice and rats and lupus nephritis in MRL/lpr mice. Many have been directed toward blocking the function of the effector $CD4^+$ T cell which has been shown to exhibit Vß isotype restriction in EAE. These approaches, including the use of anti-TCR antibodies (Acha-Orbea et al., 1988), synthetic TCR peptides (Offner et al., 1991) and superantigen treatment (Kim et al., 1991), have either successfully prevented induction of disease or reversed ongoing disease. In the case of reduction of lupus nephritis in MRL/lpr mice by SEB treatment, $Vß8^+$ $CD4^-$ $CD8^-$ peripheral T cells were reduced in a manner concomitant with disease suppression (Kim et al., 1991). In comparison, we show here that there appears to be a correlation between protection from development of EAE in PL/J mice and anergy and/or depletion of Vβ8$^+$ CD4$^+$ T cells. The staphylococcal enterotoxins have also been shown to have immunosuppressive activity i.e. suppressor cell induction (Johnson and Bukovic, 1975). Immunosuppression by SEB may also play a role in protection against EAE.

Thus, treatment of PL/J mice with SEB prior to immunization with MBP protects against development of EAE. Other studies support our conclusion and show that protection from EAE is based on the Vβ specificity of SEB as another superantigen, SEA, which is not Vβ specific does not confer protection (Kalman et al., 1993). The use of Vβ specific superantigens or their fragments that are responsible for T cell anergy and deletion as immunotherapy for experimental autoimmune disease has potential as a model for future study.

Having determined that treatment with SEB prior to immunization with MBP prevented induction of EAE, we next investigated the "flip side" of this observation, or the effect of SEB administration after immunization with MBP. We found that both SEB and SEA were capable of actually reactivating EAE post MBP immunization. There are several interesting features of superantigen induced EAE. First, we were able to induce relapses of EAE with SEB, SEB plus pertussis toxin, or SEA plus pertussis toxin. We have previously attempted to induce relapses of EAE in PL/J with MBP without success. One possible mechanism for this resistance to reinduction is an active suppression preventing re-activation of rat

MBP reactive T cells. However, with SEB alone or SEB/SEA plus pertussis toxin (but not pertussis toxin alone) we were able to overcome this effect. Furthermore, we were able to demonstrate that EAE could be re-induced multiple times, each incident occurring after an SEB injection. However, at the present time we have not observed any evidence of a spontaneous relapsing EAE in mice treated with SEB but no MBP. We suggest, therefore, that the mechanisms for suppressing the acute autoimmune illness remain intact in mice immunized with MBP, but can be overcome with SEB alone. We have also shown that reactivation of EAE is not specific for SEB. We believe that a likely explanation for the finding that SEA can reactivate EAE is that once EAE is initiated by $V\beta 8^+$ T cells, T cells with multiple specificities can be found within the inflammatory lesions in the central nervous system (CNS). Enterotoxins of various specificities can then activate these T cells with resultant cytokine release such as IL2, TNF or IFNγ. This may lead to reactivation of EAE secondary to the direct effects of these cytokines on neuronal function or to the indirect activation of T cells. Such autoreactive T cells may be specific for other CNS antigens, subdominant epitopes of MBP or may enter the lesion due to breakdown of the blood brain barrier or be attracted to the area nonspecifically by inflammatory mediators. Future studies will be directed towards clarifying these and other possible mechanisms involved.

Injection of SEB does not induce anergy in previously activated T cells. We have shown that mice previously immunized with MBP in CFA or CFA alone are resistant to induction of anergy by superantigen and that such resistance is not

MBP specific. Previous in vivo studies demonstrating the induction of anergy and apoptosis by superantigen were performed in naive, previously unimmunized animals (Kawabe and Ochi, 1990; Rellahan et al., 1990; Kawabe and Ochi, 1991). This is evidenced by the fact that PL/J mice injected with SEB 5 days before immunization with rat MBP in CFA and pertussis are protected from the development of EAE. The mechanism for the induction of anergy with staphylococcal enterotoxins is not known. In any event, changes in the characteristics of the T cells from a naive to activated cell induced by immunization seem to prevent the induction of anergy in these cells. Therefore, the timing of the administration of staphylococcal enterotoxin determines whether cells are anergized or activated.

Clinical signs of EAE could be induced in mice that previously showed no clinical signs of disease. There are several possible explanations for this. Either the initial immunization was ineffective in producing a critical threshold of MBP reactive T cells in the CNS, the T cells never reached the CNS to produce clinical disease, or the mice developed subclinical disease which was not evident on examination. Another group has attempted to induce EAE in mice previously immunized with MBP but who did not show clinical evidence of EAE, using repeated injections of pertussis toxin, MBP, or irradiation without success (P. Lehmann, personal communication). Using SEB or SEA we were able to do so. Thus, superantigen was able to initiate disease symptoms in immunized but asymptomatic animals harboring autoreactive T cells.

We believe that superantigens, such as the staphylococcal enterotoxins are capable of re-activating autoreactive T cells and inducing autoimmune diseases such as MS. It has been suggested that superantigens may contribute to the development of the relapsing and remitting cycles characteristic of various autoimmune diseases. One possibility is that individuals with a previous history of an autoimmune illness, such as MS, systemic lupus erythematosus or rheumatoid arthritis may be induced to develop acute flares of their illness following a clinical or subclinical staphylococcal infection with a superantigen producing organism. Therefore, we propose a "two hit" hypothesis in the induction of some cases of autoimmune disease. First, autoreactive T cells may be stimulated following an infection through, for example, a mechanism of molecular mimicry. No autoimmune disease may develop as either insufficient numbers of autoreactive cells are stimulated or mechanisms to suppress the proliferation of these cells develop. A second infection with a superantigen producing organism develops (which may also be subclinical) with re-activation of the autoreactive cells leading to clinical manifestations. It is also possible that a single organism may produce factors that activate autoreactive T cells, which are then further stimulated by a superantigen produced by the same organism. At the very least, superantigens appear capable of re-activating autoreactive T cells which may lead to clinical disease.

Our study and those by others (Schwab et al., 1993) using superantigens to re-activate disease in EAE or bacterial cell wall-induced arthritis, suggests that this model of MS is more complex than previously thought. For example, specific Vß8$^+$ T cells are thought to be the "culprits" in induction of EAE in the PL/J mouse, but reactivation of EAE with a non-Vß8-specific superantigen, would suggest that other factors and/or mechanisms, such as stimulation of certain cytokines may also be involved in the exacerbation of MS. Future studies will focus on what these factors may be, and such findings may provide more insight into the mechanisms of MS in humans.

Potential Therapy for Superantigen Associated Disease

In the interest of identifying potential therapeutics for superantigen activation, we demonstrate that the type I IFNs, HuIFNα and ß and BoIFNτ, inhibit the activity of several staphylococcal enterotoxin superantigens, including SEB, SEA and TSST-1. Inhibition by the type I IFNs was approximately 50 % at 1000 units/ml and occurred in a dose dependent manner. Studies of IL-2 production by T cells stimulated with SEB in the presence of the type I IFNs showed that levels of IL-2 were reduced by treatment with type I IFNs. Inhibition of Vß specific T cell proliferation by the type I IFNs was observed. Others have shown that IFNß inhibits T cell mitogenesis induced by nonspecific lectins (Noronha et al, 1993). Thus, the ability of type I IFNs to exert a potent antiproliferative effect in the face of superantigen activation makes these cytokines potential candidates as therapy for superantigen associated disease.

It is demonstrated in this dissertation that bacterial superantigens are capable of reactivating EAE, the animal model for MS. That superantigens possess this function suggests that they are important environmental factors that may contribute to the relapsing-remitting nature of MS and other autoimmune diseases such as RA and systemic lupus erythematosus. Concurrent with our observation on the effect of superantigens in EAE, it has been shown in clinical trials that IFNß suppresses exacerbations of MS (IFN-ß Multiple Sclerosis Study Group, 1993). Our data suggest one of the possible mechanisms by which IFNß may exert its ameliorating effect upon MS relapses. Considering that bacterial superantigens are ubiquitous environmental factors, regular administration of IFNß may greatly dampen the response of an individual exposed to these extremely potent immunostimulatory molecules. Such amelioration may occur by the following: 1) a reduction in the Vß specific T cell activation induced by superantigen 2) a reduction in the massive quantities of exacerbatory cytokines, such as TNF-α and IFNγ, known to be produced upon superantigenic stimulation, and 3) induction of immune suppression, such as suppressor cells, by IFNß (Noronha et al, 1990). Therefore, IFNß may prove to be regulating the exacerbatory effects of such environmental factors as superantigens.

Our study has also demonstrated the inhibitory effects of IFNs α and τ. Previous clinical trials for treatment of MS with IFNα have been inconclusive although difficulties with toxicity have been observed with both IFNs α and ß (Knobler et al., 1984; Johnson et al., 1990; Kastrukoff et al., 1990). We show that IFNτ lacks the toxicity exhibited by the other type I IFNs as tested *in vitro*. Studies presently underway in animal models show the consistent lack of toxicity of IFNτ and the cDNA of the human counterpart of BoIFNτ has been identified (Whaley et al., 1994). When tested in combination, the type I IFNs were no more effective inhibitors than when tested individually, suggesting that the mechanism by which the IFNs exert their antiproliferative effect is different from their toxic effect. The remarkable lack of toxicity exhibited by IFNτ at very high doses suggests it to be a most attractive candidate for testing as a potential therapy for autoimmune diseases such as MS.

In addition to their role in autoimmune disease, the staphylococcal enterotoxin superantigens induce multiple maladies including toxic shock syndrome and food poisoning (Bergdoll et al., 1981). New potential superantigens have also been identified and shown to be produced by a number of organisms among which are *Yersinia, Clostridium,* and *Mycoplasma* (Cole et al., 1990; Bowness et al., 1992; Uchiyama et al., 1993). Recent evidence clearly points to the superantigens produced by these organisms as the culprits in many of the diseases for which they are responsible. Other diseases such as Kawasaki syndrome follow the same pattern of superantigen related disease. The numbers of T cells bearing Vβ2 and Vβ8 TCR are increased during the early stages of Kawasaki syndrome suggesting superantigen as an agent in the pathogenesis of this disease (Abe et al., 1992). It is likely that the inhibitive effects of the type I IFNs we have shown here for the staphylococcal enterotoxin superantigens will hold true for the more recently described superantigens. Thus, the type I IFNs may prove to be of great therapeutic value for a range of superantigen associated diseases including toxic shock syndrome and Kawasaki syndrome.

The myriad of activities ascribed to the IFNs have led to their use as treatment for several conditions including cancer and hepatitis. We have demonstrated that the type I IFNs are able to significantly inhibit superantigen stimulation. The potent antiproliferative activities of the type I IFNs upon superantigen activation strongly suggests their further study as therapy for superantigen associated diseases.

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BIOGRAPHICAL SKETCH

Jeanne Margaret Soos was born on April 5, 1967 at Rahway Hospital in Rahway, New Jersey (Exit 132 off the Garden State Parkway) to Steve and Betty Soos. She completed her primary education at St. Michael's Byzantine Catholic School in Perth Amboy, New Jersey, where her teachers, Sister Theodosia and Mrs. Van Brummlen, motivated her to strive for her full potential. The Soos family lived in New Jersey until 1981 when they moved to Boca Raton, Florida. They shared a few happy years together until the loss of Jeanne's mother. She and her father tried to cope and after her teenage years came to truly appreciate each other. During that time, she graduated from Spanish River High School in 1985 as cosalutatorian. She went on to the University of Florida to study microbiology and cell science and received a Bachelor of Science degree in 1989. Her interest in immunology intensified as an undergraduate, so she entered graduate school in the Department of Pathology and Laboratory Medicine, Program in Immunology and Molecular Pathology, where she was greatly influenced by Dr. Howard M. Johnson and Drs. Carlo and Giovanella Moscovici. As a first year graduate student, she made an important co-discovery with her fellow first year graduate student, Brian Szente, this important discovery being each other. Their first date was the 1991 Department of Pathology Graduate Symposium Dinner, and they 27-

were subsequently married on July 17, 1993. After completion of her doctoral program, she plans to pursue research in the areas of neuroimmunology and autoimmunity.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Howard M Johnson

Howard M. Johnson, Chairman
Graduate Research Professor of
Pathology and Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maureen M. Goodenow
Assistant Professor of Pathology and
Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edward M Hoffmann

Edward M. Hoffmann
Professor of Microbiology and Cell Science

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Ammon B. Peck
Professor of Pathology and
Laboratory Medicine

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Joel Schiffenbauer
Associate Professor of Immunology and
Medical Microbiology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April, 1994

Dean, College of Medicine

Karen G. Holbrook
Dean, Graduate School

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